

LILLEMOR LEWAN

A STUDY OF MOUSE LIVER REGENERATION  
AT THE ORGAN, CELLULAR AND NUCLEAR LEVEL

LUND 1972





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AKADEMISK AVHANDLING

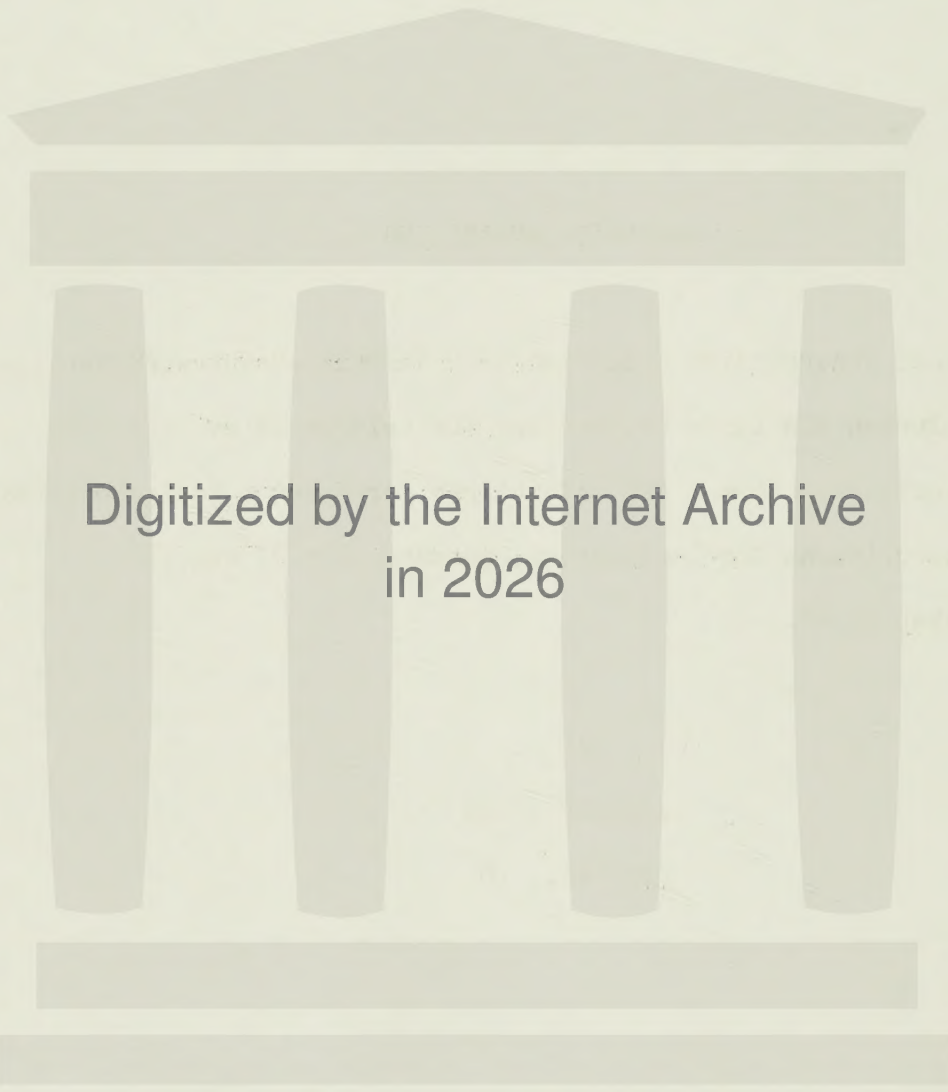
som med vederbörligt tillstånd av Matematisk-Naturvetenskapliga  
Fakulteten vid Lunds Universitet för avläggande av filosofie  
doktorsexamen kommer att offentligen försvaras i Zoofysiologiska  
Institutionens föreläsningssal, lördagen den 27 maj 1972  
klockan 10.00.

AV

LILLEMOR LEWAN

FIL.MAG., LD

LUND 1972



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A study of mouse liver regeneration at the organ,  
cellular and nuclear level.

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Institute of Zoophysiology

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Sweden.

Lund 1972

A study of some of the  
principles and methods of

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The purpose of this study was to analyse the changes in nuclear proteins which occur during mouse liver regeneration and their influence upon the regenerative process. In the course of investigating this complex system, questions arose which demanded a study of related factors, including nuclear proliferation, structural changes in the liver cell, and variations in the cellular content of nucleic acids and polyamines. The principal findings of each part of the study are mentioned in the summaries of the relevant papers, listed below, which are bound in this volume. The following pages will be devoted to a discussion of the general methodological problems associated with the investigation of nuclear changes during a complicated process of growth.

1. Changes in the weights of total body, and of liver and other organs in mice after partial hepatectomy (Lewan, in manuscript).
2. Protein, nucleic acids and cell structure in the regenerating mouse liver (Lewan, 1972. Z. Zellforsch. in press).
3. Putrescine and polyamines in relation to nucleic acids in mouse liver after partial hepatectomy. (Heby and Lewan, 1971. Virchows Arch. Abt B 8, 58-66).
4. Nuclear proliferation and the amount of DNA per parenchymal nucleus during mouse liver regeneration (Lewan, 1971. Virchows Arch. Abt B 9, 280-291).
5. The amount of DNA per nucleus during mouse liver regeneration. (Lewan, in manuscript).
6. The stathmokinetic effect of vinblastine during mouse liver regeneration. (Lewan, in manuscript).
7. Protein content of liver cell nuclei isolated after partial hepatectomy. (Lewan, in manuscript).



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"If there were no regeneration there could be no life. If everything regenerated there would be no death. All organisms exist between these two extremes." Goss, 1969.

## Introduction

Despite intensive study and considerable knowledge of some aspects, the fundamental reasons for the initiation of restorative liver growth and its regulation after partial hepatectomy are still unknown (7, 13, 26, 72, 74, 85).

Owing to its regenerative capacity, the liver offers a suitable system for the study of nucleic acid and protein metabolism (9, 11, 12, 35) and, because of its special features, also of lipid metabolism (23, 28, 29). The relation of metabolic changes to gene activity is an interesting problem, and the DNA synthesis and mitotic activity during the rapid liver growth invites studies on the cell cycle (3, 8, 21, 22, 32, 68, 69) and the mitotic process (5, 62). Remarkable indeed is the possibility that one third of the liver is not only able to fulfill the duties of a normal liver, but can also simultaneously renew itself (6, 7). Also, the rapid regenerative growth of the liver offers possibilities for comparison between the well regulated normal growth and the defective, uncontrolled growth of tumours (24).

However, the regenerating liver is a complicated system, the study of which will easily tend to be a registration of artifacts. Therefore in this study, some essential constituents of the liver and features of the growth process have been followed. I believe that quantitative registrations are a necessary prerequisite for qualitative studies and for incorporation experiments during the growth process. Moreover





processes observed in the liver after partial hepatectomy should be integrated with those occurring in other parts of the body.

Experimental animals. Most studies on liver regeneration are made on rats (9, 11, 12, 35). In this study, however, the mouse was chosen as the experimental animal. The characteristic events of the regenerative process, DNA synthesis and mitosis, start later after partial hepatectomy in the mouse (44) than in the rat (31). Therefore the different reactions bringing about the restorative growth and the changes they confer upon the concentrations of the tissue constituents should be easier to separate as regards time in the mouse than in the rat. Moreover, the effects of the operation should be more drastic in the mouse, as the resected 2/3 of the liver amounts to 4-5% of the body weight (paper 1) as compared to 2-3% in the rat (38). On the other hand this difference in loss of functioning liver tissue probably contributes to a greater sensitivity to the surgical process in the mouse than in the rat.

Control animals. Analysis of sham operated (paper 2) animals and hepatectomized animals should be made at the same intervals after the operation and compared to the analysis of normal animals. This makes possible a correction for the effects of anaesthesia and operative stress as illustrated in paper 7.

Anaesthesia. The anaesthetic used was Nembutal (10 mg/ml 0.9% NaCl) given intraperitoneally, 6  $\mu$ l per gram body weight. Sham operated animals were unconscious for 30-60 minutes, the hepatectomized animals for about two hours. As the operation takes only about ten minutes, ether anaesthesia being of shorter duration, could have been used (77). However, ether anaesthesia was found to be more difficult to regulate at a constant level.

Nembutal is known to have various metabolic effects and causes a proliferation of smooth endoplasmic reticulum in the liver (25, 52, 65). Thus, part of the increased amounts of cytoplasmic membranes found after hepatectomy (paper 2) may have been caused by the anaesthetic.









obtained when the percentage increase in liver weight was based on liver weight at operation, calculated on body weight than when liver weight at operation was calculated on the weight of the resected liver. This may depend on the greater blood content of the liver resected at operation than of the liver dissected from a decapitated animal. The comparison in Fig. 1 shows that the percentage increase in liver weight in this (papers 1,2,3) study should not have been overestimated.

Synchrony of the regenerative process. The criteria of a successful regeneration used here are the healthy appearance of the animal (77) and a not too pale colour of the regenerating liver at stages later than 48 hours after the operation. The healthy looking animals can, however, have very varied regenerated liver weights at the same time interval after operation, Fig. 2A. Their body weights also differ a great deal, Fig. 2B.

The results in paper 1 indicate that the weight of each operated animal should be followed after the operation so that the resumption of the daily rhythm between 36 and 48 hours after hepatectomy can be used for selecting animals in a synchronized healthy postoperative state.

The mitotic index (60, 71, 86) and the labelling index (2, 15, 44, 60) are the most reliable methods for analysing the synchrony of the regenerative process. They are, however, time consuming processes. If there is a correlation between labelling and mitotic indices and the body weight changes after hepatectomy (42, 58, 71), the synchrony of the regenerative process in the liver could be easily controlled by body weight registrations.

The percentage amount of dry matter in the liver of the rat has been found to decrease when the mitotic index increases (88). This is indicated in this study also, Fig. 3 (c.f. paper 1 and papers 3, 5, 6). Therefore determination of the dry matter content of the liver may aid in evaluating the synchrony of the regenerative process.



Registration of tissue components. There are three main possibilities of presenting the composition of the regenerating tissue. The different constituents may be presented as A) per gram of tissue B) per organ C) per cell D) per DNA E) per protein. I have chosen to relate the concentration of the liver constituents during regeneration as per gram of liver tissue. This is the primary result at analysis and the reader may make his own calculation based on this value.

In order to demonstrate the increase in the different constituents in the total liver during the growth process, the concentrations may easily be multiplied by the weight of the regenerating liver (1, 37, 82). Pre-supposing that the animals used are of the same sex and of very similar weight, this can give an accurate picture of the changes in the liver. If, however, animals of both sexes are used, and their body weights are not similar, the presentation of tissue composition per organ necessarily involves the more uncertain calculations of percentage increase in liver weight as described above and in papers 1, 2 and 3.

The illustration of tissue composition per cell is unsuitable in the liver due to the presence of both parenchymal and non-parenchymal cells (16), and also to the large numbers of binucleate cells (27).

A presentation of the results of analysis per nucleus has been suggested (67). The estimation of the number of nuclei in the liver is, however, rather uncertain (papers 4 and 5).

The possibility remains of calculating the results per DNA, the concentration of which in weight per wet weight, is fairly constant during regeneration (paper 2). However, the concentration of protein is even more constant in weight per wet weight (paper 2).

Mitotic activity and polyploidy. The mitotic process after partial hepatectomy has been studied intensively, as has the degree of polyploidy in the regenerating liver. Normal mitoses, amitoses and nuclear fusion of interphase nuclei have been reported. However, it is now considered that much of the mitotic activity after partial hepatectomy does not lead to





proliferation of nuclei but rather to an increased polyploidy because of fusion of the chromosome groups during the mitotic process (35). This assumption is based upon observations in cell suspensions (62), and is supported by microspectrophotometric analysis of liver sections from the rat (0-54 hours) (34) and the mouse (0-96 hours) (55) at various intervals after hepatectomy. The results given in paper 5 are in agreement with this. During later stages of the regenerative process the increased polyploidy, as indicated by biochemical measurements (53, 82) is also found by karyometric analysis (43, 62). The nuclear volume increases after hepatectomy, and the good correlation found between nuclear volume and DNA content in the normal liver (20) is disturbed between 20 and 42 hours after hepatectomy in the rat (34). In a preliminary study a similar disturbance is indicated in the mouse liver between 30 and 55 hours after hepatectomy (41).

The number of nuclei in the liver. The results presented in paper 4 regarding the number of nuclei per gram of liver as determined in homogenates during regeneration is a confirmation of previous analyses (Fig. 4). The animals in series C had regenerated their livers to 80% at 140 hours after operation. The normal animals and those analysed 140 hours after hepatectomy in series D were from the same litter with body weights similar to those in series C. They appeared to be very healthy 140 hours after operation and at autopsy their livers had a normal colour. As the DNA concentration during mouse liver regeneration showed only minor variations (paper 2, 3) these results indicate that the degree of polyploidy increased by more than 100% and that hardly any proliferation of nuclei had occurred. The animals whose livers were analysed by section technique at 140 hours after hepatectomy (paper 5) all had body weights similar to those of the animals analysed in series C and D and had regenerated their livers to between 80 and 100%. However, the analysis on sections revealed no decrease in the number of nuclei per volume of liver during regeneration. Thus, as



the biological differences were small, it is probable that the differences found 140 hours after hepatectomy, when determining the number of nuclei per volume or gram on sections and in homogenates, are caused by technical rather than biological factors.

Membrane changes. After hepatectomy the fatty acid composition of the phospholipids of the liver changes in a manner which, if pronounced enough, can give rise to disturbances in the permeability properties of membranes (23). Also the intracellular concentrations of  $\text{Na}^+$  and  $\text{P}_i$  increase (57) and the electrophoretic mobility of the nuclei changes (49) after partial hepatectomy. Therefore alterations may occur in the cell membrane system contributing to the different results found between normal and regenerating liver at homogenization (paper 4). It is, however, probable that the differences have been exaggerated in the buffer chosen.

Nuclear proteins. The histones or nuclear basic proteins have long been considered to be of importance for gene regulation (45). Therefore many studies have been carried out on their synthesis (39, 40, 64, 70, 78) and modification (63, 66, 76, 81) during the regenerative process. Within the nuclei there are also non-histone proteins, some of which are acidic. Their acidic character is determined by their high content of acidic amino acids (61, 73, 75) and also by a high content of phosphate-groups (54). The total amount of protein found within the nuclei is largely a function of the isolation method employed (14, 18, 19, 36). However, a neutral soluble, an acidic soluble and an alkali soluble protein fraction are found (4). Acidic proteins are found in the neutral soluble protein fraction which mainly constitutes the "nuclear sap" fraction. Acidic proteins are also found in the alkali soluble fraction together with DNA (4). The acid soluble protein fraction should mainly be constituted by histones.

The acidic proteins in the alkali soluble fraction can be called chromatin acidic proteins. For determination of the "activity" at incorporation experiments or for quantitative determination of the amount of acidic chromatin proteins, they may be analysed in alkaline solution, or





recovered as an insoluble residue after the extraction of DNA from the chromatin with PCA (39). However, for electrophoretic experiments, the acidic proteins must be separated from DNA using milder methods. This can be brought about in 5 different ways, and nuclei or isolated chromatin can be used for the preparations.

1. On extraction with 1-2M NaCl the chromatin will dissolve. Dialysis of the extract against 0.14M NaCl will cause DNA and histone to precipitate and some acidic proteins will remain in solution and can be used for electrophoresis (59, 83).
2. On extraction with 4M CsCl, the chromatin will dissolve. At centrifugation of the solution, DNA will sediment and some acidic proteins will remain in the supernatant and may be recovered for electrophoresis (10).
3. Gel filtration of the chromatin extracted by the use of highly concentrated salt solutions results in a separation of the DNA and protein (56).
4. Some acidic proteins may be separated from the chromatin by the action of detergents and urea in the presence of reducing agents (61, 33).
5. The chromatin acidic proteins may be liberated from the DNA by the use of phenol, based originally on the methods of Kirby (48, 73).

During preliminary experiments using modifications of these methods, the extraction with CsCl followed by separation of DNA and protein by centrifugation seemed to be the most successful. The result is mentioned in paper 7.

The increased synthesis of acidic nuclear protein during stimulated growth (4, 39, 51, 73, 75, 79) indicates a possible role for these proteins at gene regulation and this is supported by an observed tissue specificity of the proteins (50, 51, 59, 80) and their promoting of RNA synthesis (4, 17, 46, 47, 80, 84). Thus, according to the model of Baserga (4) for the isoproterenol stimulated salivary gland and also supported by Becker (7) some reaction may occur at the cell membrane after partial



hepatectomy, causing the loss of some messenger RNA inhibitory molecule from the cell. An acidic protein is synthesized by means of the activated messenger RNA. The acidic protein enters the nucleus and activates the synthesis of RNA on a specific part of the genome. Thus, in this way the series of events leading to the restorative growth of the liver may be initiated.





1. Weight of regenerating liver as per cent of liver weight at operation, calculated using two different methods. Swiss albino and C57 mice.

(-○-○-○-) Liver weight at operation, calculated, using body weight.

(●-●-●-) Liver weight at operation, calculated, using weight of resected lobes.

Each point represents 5-10 mice. Mean  $\pm$  S.E.

Fig. 2. Changes in liver and body weight after partial hepatectomy.

Each point represents one C57 mouse.

Fig. 3. Dry matter content (w/w) in livers from Swiss albino (○) and C57 (●) mice after hepatectomy. The arrow indicates when mitotic activity starts. Each point represents 4-6 mice.

Mean  $\pm$  S.E.

Fig. 4. Number of nuclei per gram of regenerating liver in four series as per cent of the number in normal liver. Determination in homogenates. Each point represents one C57 mouse. Series A (△) and B (▲) were homogenized in TKM-buffer according to paper 7. Series C (○) and D (●) were homogenized in citric acid, containing  $\text{Ca}^{++}$  according to paper 4.



Fig. 1

Liver weight per cent of normal

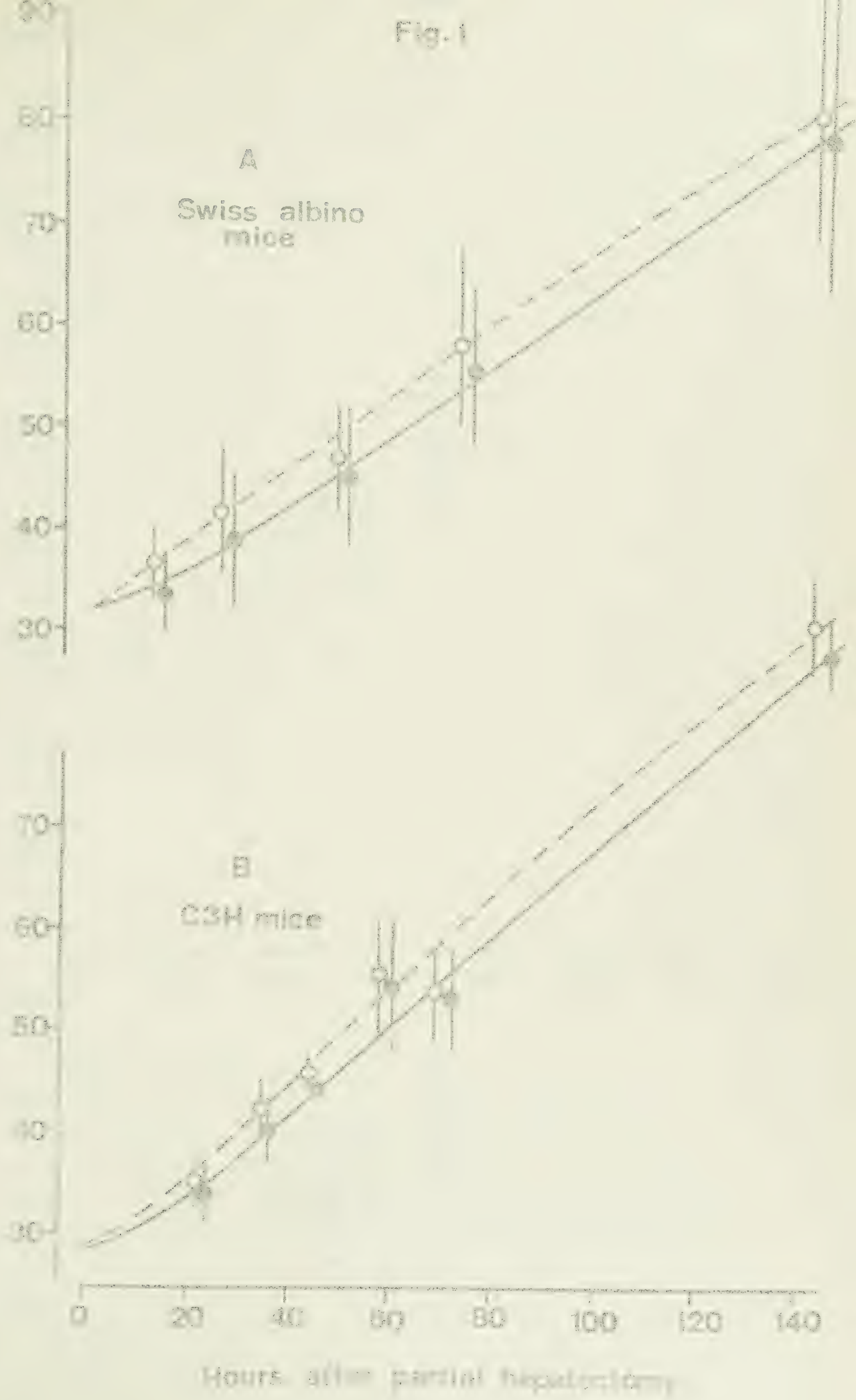




Fig. 2

Liver weight per cent of normal

90  
80  
70  
60  
50  
40  
30  
20  
10

A

body weight, per cent of  
weight at operation

100  
90  
80  
70

0 20 40 60 80 100 120 140

B

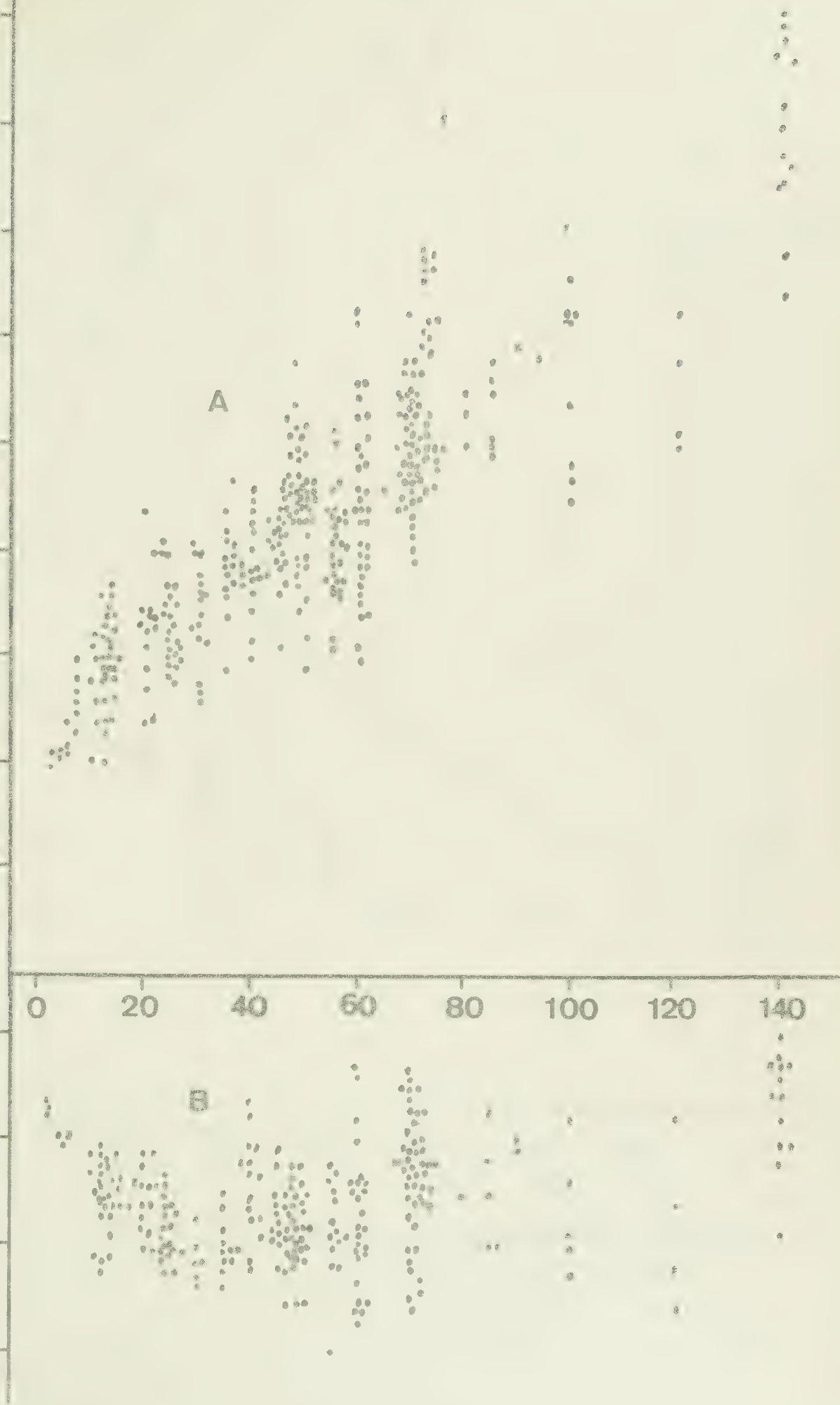






Fig. 3

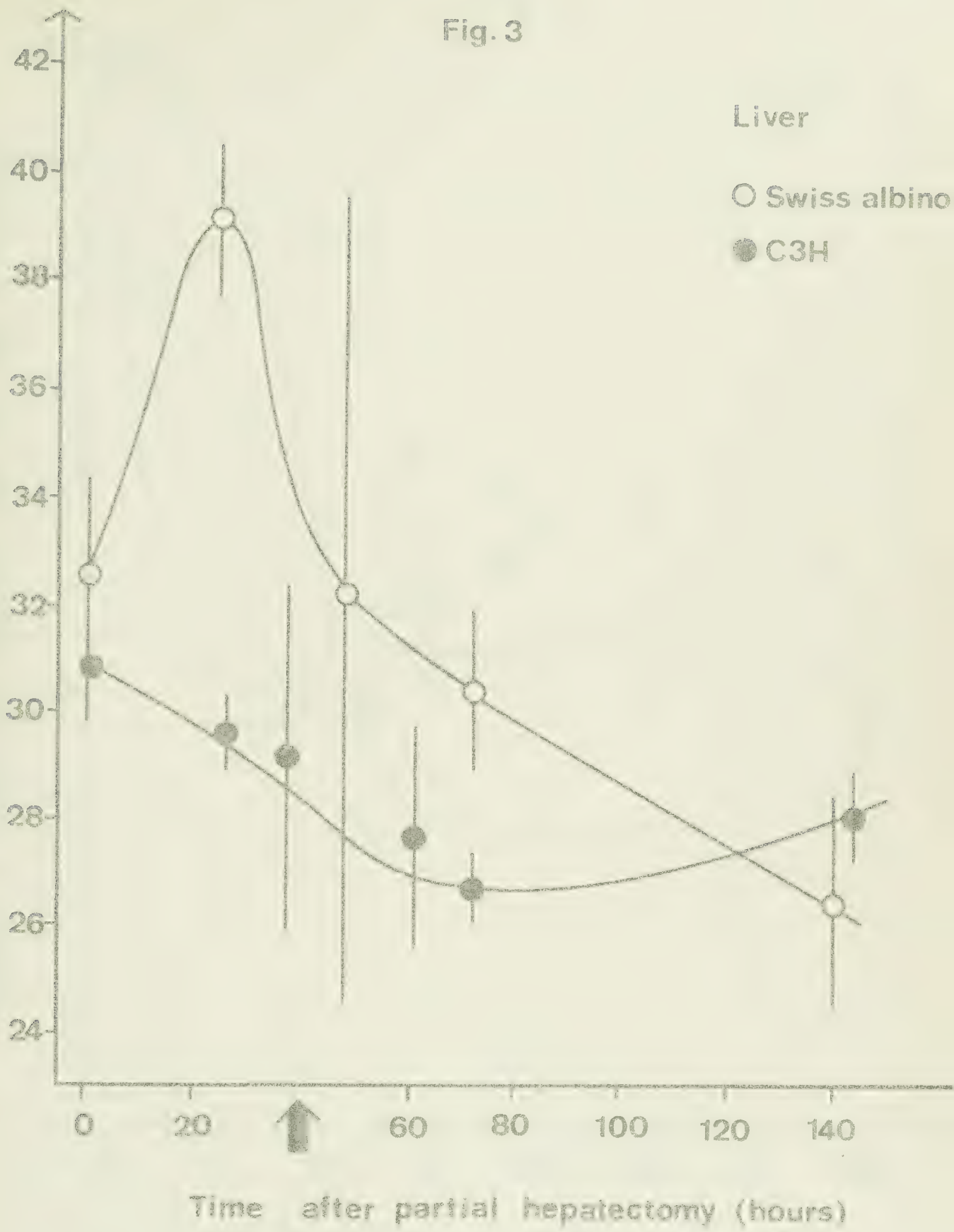
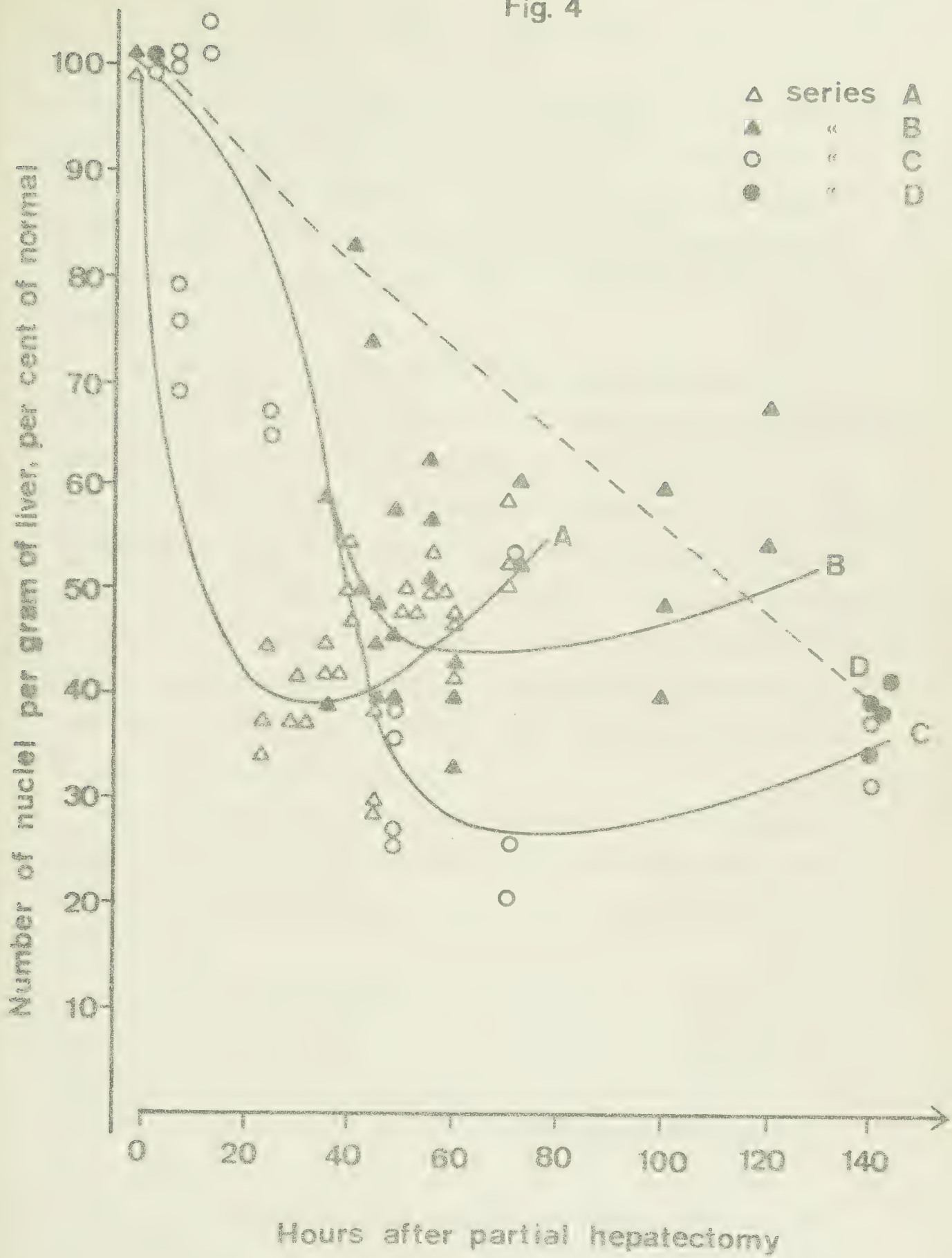




Fig. 4





General summary and conclusions

1. The liver weight increases after partial hepatectomy as opposed to the weight of other organs and the total body weight and contrary to after a sham operation when the liver weight decreases. The early weight increase in the liver after hepatectomy is partly dependent upon changes in the distribution of water and fat, and possibly other products, among the tissues of the body.

The recovery of the mouse after partial hepatectomy can most probably be registered as the resumption of the diurnal rhythm of body weight at the time when DNA synthesis and mitotic activity in the liver increase.

2,3 The concentrations of liver constituents during regeneration depend on their production and degradation within the liver and on their influx and efflux from the liver, and also on the total liver mass. As the amount of liver increases during the regenerative process, an increased concentration of a given substance (putrescine, spermidine, RNA) must depend on the fact that its production within the liver or influx to the liver is larger than its degradation or efflux.

A decreased concentration of a given substance (total protein, spermine, DNA) indicates that its production or influx does not compensate for its degradation or efflux from the liver and for the increase in total liver mass.

Variations in the concentration of a given substance (soluble and insoluble protein, putrescine, DNA) noted during the growth process indicate changes in the balance between, on the one hand, production or influx of the substance and on the other hand its degradation or efflux and the increase in liver mass.

Increased concentrations of some liver constituents (RNA and spermidine) and decreased concentrations of others (total protein, spermine, DNA) may be an advantage for or even a stimulus to the growth process. However, the variations found may also be unescapable results of changes in the liver following resection.





The measured concentrations of soluble components are most likely to be influenced by the stability of the liver tissue at homogenization and changes in the lipophilic properties of the liver tissue.

4,5,6 The nuclear proliferation during mouse liver regeneration is a complicated process, probably not simply related to the mitotic activity as the mitoses may bring about increase in polyploidy rather than proliferation of nuclei.

The number of nuclei in the regenerating liver is not easy to determine with certainty, due to the technical difficulties in obtaining similar results when analyses are made on sections and on homogenates. It should be possible to overcome these difficulties by analysing large, thick sections, and by checking the volume changes in the specimen during preparation. A possible change in the number of nuclei fragmented at homogenization will be revealed if the number of nuclei and the amount of DNA are determined in homogenates before centrifugation and in the supernatant and pellet obtained after adequate centrifugation.

The number of nuclei entering mitosis per hour was found to be large both at 48 and at 72 hours after hepatectomy. There were large individual variations. The mitotic index at 140 hours after hepatectomy was still elevated as compared to that found in normal animals.

7 A study on the changes in the nuclear proteins and their influence upon gene activity during mouse liver regeneration, should start with a selection of experimental animals in a synchronized healthy state after hepatectomy and a selection of livers with similar dry matter content at the time of analysis after the operation.

The proportion between different types of nuclei should be analysed on sections and in homogenates and in the suspension of isolated nuclei to be analysed, to ensure that no selection of nuclei occurs during the preparation.



Attention should be paid to the possible influence on the analysis of variations that occur in the proportions between the different nuclei in the liver during the regeneration

Care should be taken that the results obtained regarding nuclear proteins are not merely due to general changes in the composition of the liver tissue during regeneration.

Qualitative comparisons may reveal similarities between liver chromatin acidic proteins, total nuclear proteins, cellular proteins and serum proteins.



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Changes in the weights of total body and of liver and other organs in  
mice after partial hepatectomy<sup>x</sup>

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Page Heading: Weight changes after partial hepatectomy



### Summary

After partial hepatectomy in mice the total body weight and the weights of the heart, the kidneys, the spleen and the thymus decreased, while that of the liver increased. The daily rhythm of the body weight was disturbed. When DNA-synthesis and mitotic activity were occurring in the liver, the body weight started to increase and reassume daily rhythm. These changes were followed by a rapid restoration of the weights of the thymus and spleen especially. The curve for the dry matter content of the spleen and the kidneys reached a maximum whereas that of the liver reached a minimum at the period for mitotic activity in the regenerating liver.

The increase in weight of the liver during its premitotic period (0-36 hours after operation) may depend upon the accumulation of degradation products originating from other parts of the body. The weight changes found in the thymus and the spleen may indicate a repression of DNA-synthesis during early liver regeneration and, during recovery, a resumption of DNA synthesis in several of the proliferating tissues.

The different sensitivity and regeneration capacity of tissues, indicated by the weight changes in the organs, may be of importance at the derepression of the DNA synthesis and mitotic activity in liver parenchymal cells after hepatectomy.

### Introduction

During liver regeneration after partial hepatectomy there is a rapid increase in liver weight. At first the parenchymal cells hypertrophy and at about 20 hours after the operation (for the rat) or 30 hours (for the mouse) DNA synthesis and mitotic activity commence (Bucher, 1963, 1967; Grundmann and Seidel, 1969; Bengmark, 1970).



Very early after partial hepatectomy the processes in the animal lead to an increase in liver weight. The animal itself, however, must be in bad condition, as revealed by the decrease in body weight (Higgins and Andersson, 1931; Tsuboi et al., 1954). In order to illustrate the change from a catabolic into an anabolic state in mice after partial hepatectomy, weight registrations of the total body, the liver, the thymus, the spleen, the heart and the kidneys were made 0-140 hours after partial hepatectomy. Furthermore, the dry matter content of the liver, the kidneys and the spleen was analysed.

It is well known that several parameters in normal and regenerating liver are subjected to a daily rhythm (Jaffe, 1954; Barnum et al., 1958; Russo and Llanos, 1964; Nash and Llanos, 1969). Therefore attention was paid to diurnal variations in body weight.

#### Material and methods

Experimental animals. Young male and female C3H mice from an inbred strain, kept at the institute, were used for the main experiments, the male weighing 18-20 g, the female 15-16 g. Body weight registrations were also performed on one year old male mice. The influence of a sham-operation was analysed in a group of male Swiss albino mice weighing 29-30 g, from Anticimex, Uppsala. All animals were kept under standard conditions regarding light and temperature before the operation, but were kept in the laboratory after the operation. They were given a standard laboratory feed and were allowed to eat and drink ad libitum.

Partial hepatectomy. Partial hepatectomy, essentially according to Higgins and Andersson (1931) and sham-operation were carried out as described previously (Heby and Lewan, 1971).

Autopsy. The animals were killed by decapitation at intervals upto 140 hours post hepatectomy. This is illustrated in Figures 1-3. The organs to be investigated were dissected out and weighed immediately.





Weight registrations. The mice were weighed at operation and at autopsy. The body weight at autopsy was expressed as a percentage of that at operation minus the weight of the cut-away liver lobes. The weight of the regenerating liver was expressed as a percentage of the liver weight at operation as described earlier (Heby and Lewan, 1971). The course of the liver weight at 24 and 36 hours after hepatectomy was also calculated on the assumption that the catabolism in the liver was as large as that found in the kidneys after the operation. Correction for the dehydration of the kidneys was made.

The weight of the thymus was expressed as a percentage of the mean thymus weight in a group of ten control mice,  $49.6 \pm 6.3$  g.

The weights of the heart, the kidneys, the spleen and the liver in normal mice were each correlated with the weight of the total body, using linear regression analysis. The weight of the liver in sham operated C3H mice and the weights of the heart, the kidneys and the spleen in hepatectomized animals at operation were calculated from the found correlations. The weights of the organs at autopsy were expressed as percentages of the weights at operation.

Circadian rhythm of body weight. Registrations of body weights were made between 8 and 10 a.m. and between 6 and 8 p.m. on 5 unoperated old mice and on 5 unoperated young mice for 3-4 days. The young mice were shamoperated and weight registrations carried out during a further 6 days. Registrations of body weight were also performed on 5 hepatectomized young mice for 6 days.

Dry weight determinations. Small test tubes containing sand were filled with wet tissue, which was minced with the sand using glass rods and dried at  $105^{\circ}$  in an oven. Weight registrations before and after the drying procedure gave the amount of dry matter content per gram of wet weight in the tissue.



## Results

Bodyweight (Fig. 1). The body weight of the analysed animals was subjected to a diurnal rhythm. The rhythm was more pronounced in young animals than in old. It was abolished during the first day after partial hepatectomy but not after a shamoperation. The body weight attained its lowest values 36 and 60 hours after a hepatectomy. The loss after a shamoperation amounted to 12% of the preoperative body weight and 14% after a partial hepatectomy. The latter figure amounted to 19% if the weight of the cut away liver pieces was counted as a loss in body weight.

Dry matter content (Fig. 3). The dry matter content of the regenerating liver decreased to a minimum around 50-70 hours after hepatectomy, whereas that of the spleen and kidneys increased to a maximum at this time.

These changes in dry matter content around 48 hours after hepatectomy correspond to a weight loss by dehydration to 88% of the weight of the spleen and 95% of the weight of the kidneys at operation. In the liver it corresponds to a weight increase by water inflow to 116% of the preoperative liver weight. Thus the regenerating liver would have increased from 28 to 33 per cent of the preoperative liver weight merely by the influx of water.

Organ weights (Fig. 2A and B). The coefficients for the linear correlations in normal C3H mice between the weights of various organs and the body were as follows; heart:body 0.78; kidneys:body 0.95; spleen:body 0.83 and liver:body 0.71. The coefficients for the linear regressions between the weight of the body and the weights of the organs within the group of normal Swiss albino mice were less and 0.5.



The weights of all the analysed organs, except that of the liver, decreased after partial hepatectomy. The percentage decrease in weight of the heart and the kidneys was somewhat larger than that of the total body. The percentage decrease in weight of the spleen and especially the thymus was considerably larger than that of the total body. All organs attained their minimal weights at about the same time as that of the total body weight, around 48 hours post hepatectomy.

If the catabolism in the liver after partial hepatectomy were as large as that in the kidney, the liver weight should have decreased from 28 to 23 percent of the normal liver weight at 36 hours after the operation, Fig. 2A.

The liver weight decreases after a sham-operation, Fig. 2A, Table I. Weight changes in the other organs similar to those occurring after hepatectomy, though less pronounced, were indicated in sham-operated animals, Table I.

### Discussion

There was obviously a turn from a catabolic into an anabolic state in the mice around 40 hours after partial hepatectomy, when both DNA-synthesis and mitotic index are known to increase in the liver (Bade et al., 1966; Heby-Lewan, 1971; Fig. 1). The stress on the animals at operation and afterwards may have brought about a general depression of DNA-synthesis in the body, followed by its resumption during recovery. Furthermore, the greater sensitivity of some tissues (cf. fig. 2A and B) may hold true also within the tissues of the liver and be of importance for the derepression of DNA-synthesis and mitotic activity in the parenchymal cells.

Decreased thymus weight is also found after hydrocortisone treatment (Takashi and Hoshino, 1961), X-irradiation (Smith and Kiefer, 1957) and in late carcinogenesis (Edwards et al., 1971). It is associated with decreased DNA synthesizing capacity and efflux of cells (Knecht and Ertl,





1969; Edwards et al., 1971). Decreased spleen weight is found after treatment with stathmokinetic agents and is also associated with decreased DNA synthesis (Lancker et al., 1966). The decrease in thymus and possibly spleen weight observed may therefore indicate a general repression of DNA synthesis and mitotic activity in the body during early liver regeneration, and may also indicate increased levels of glucocorticoid hormones. These hormones are known to suppress somatic growth and inhibit liver DNA-synthesis in young rats (Henderson, 1971). If administered to rats after partial hepatectomy, they delay DNA-synthesis and mitotic activity (Feigelson et al., 1962; Rizzo et al., 1971).

A resumption of DNA-synthesis in several of the proliferating tissues after partial hepatectomy, may be a general sign of recovery of the animal, perhaps related to changes found in the hypothalamic secretion around 40 hours post hepatectomy in mice (Llanos et al., 1971). DNA-synthesis has been found not only in the liver parenchymal cells, but also in liver lymphoid cells (Fichtelius and Kullgren, 1971) and in the thymus regenerating after hepatectomy-induced involution in the rat (Craddock et al., 1964).

DNA-synthesis was also indicated in the spleen by its rapid weight regeneration (Lancker et al., 1966). Increased DNA-synthesis has been found in several lymphoid tissues 3 days after partial hepatectomy by Sakai et al., (1970).

As all analysed organs, except the liver, were found to decrease in weight during the first 40 hours following the operation, degradation products from several parts of the body may have contributed considerably to the early, rapid increase in liver weight (Fig. 2A). Evidence for this conclusion is found in several reports in the literature. Thus the increase in blood pressure in the liver after operation (Benaceraff et al., 1957) results in damage to the liver cell membranes and penetration of blood plasma and blood cells into the liver cells (Pfeifer and Banasch,



1968). An increased phagocytosis is also indicated after partial hepatectomy (Benceraff et al., 1955; Ledoux et al., 1967; Stern and Duwelius, 1957). Recirculating lymphocytes are abundant in the liver (Fichtelius and Groth, 1963) and their thymidine is reutilized at DNA synthesis during liver regeneration (Bryant, 1962, 1964). Also, fat accumulates in the liver very early after hepatectomy (Camargo et al., 1966; Tsuboi et al., 1954), and proteins may be deposited (Roberts, 1953). The presence of fat probably delays the decrease in the dry matter content of the liver, Fig. 3. A water oedema would otherwise have been an early result of the change in blood pressure at resection.

Unless products from other parts of the body had accumulated in the regenerating liver, its weight may have decreased to the same degree as that of the kidneys, Fig. 2A. This has been indicated in the figure by a dotted line for liver weight. The early synthesis of RNA and protein (Rabes and Brändle, 1969) known to occur in the regenerating liver probably makes liver catabolism less severe than that of the kidney after hepatectomy. However, the course of the liver weight after a sham operation indicates that the synthesizing capacity was not large enough even in the intact liver to eliminate the weight decrease caused by an operation, Fig. 2A, Table I. A sham operation or stress situation also increases RNA synthesis in the liver (Tsukuda and Lieberman, 1965; Majumdar et al., 1967).

The thymus regenerates its weight after stress induced involution but not after partial resection. This may depend upon a disturbed proportion between the tissues in the thymus after stress, but not after partial resection (Borum, 1969). After partial resection of the liver, there are indications for a slight change in the proportion between parenchymal and non-parenchymal cells (Edwards and Kock, 1964; Wilson et al., 1953). The decrease in the number of non-parenchymal cells

et al., 1953). The decrease in the number of non-pregnant cells

is not statistically significant.

The results of the present study are in agreement with those of

but not other partial reactions. This was based upon a listing

for between the changes in the degree of other signs, but not other

parameters of the experimental cells (Harrison and Kohn, 1954; Wilson

1954).

during the premitotic period of regeneration may possibly release the parenchymal cells from repression and thus bring about induction of DNA-synthesis and mitotic activity during the recovery.



Fig. 1. The body weights in groups of 5 C3H mice expressed as percentage of the weight on the first morning. Mean  $\pm$  S.D.

Fig. 2A. The weight of heart, kidneys and liver after hepatectomy and the weight of liver after sham-operation in C3H mice expressed as percentage of the weights at operation. Mean  $\pm$  S.D. Each point represents 4-6 animals. The curve for liver weight, calculated on account of weight decrease in the kidneys, is represented by the dotted line.

Fig. 2B. The weight of spleen and thymus after hepatectomy in C3H mice, expressed as percentage of the weights at operation. Mean  $\pm$  S.D. Each point represents 4-6 animals.

Fig. 3. The dry matter content in per cent of wet weight in liver, spleen and kidneys of C3H mice after partial hepatectomy. Mean  $\pm$  S.D. Each point represents 4-6 animals.

Table I. The body weight and the weights of thymus spleen, heart and kidneys in Swizz albino mice after sham-operation. Mean  $\pm$  S.D.





Table I. Body and organ weights after shamoperation.

| Hours after<br>shamoperation | n | Weight of <sup>x</sup><br>body | Wet weight <sup>x</sup> in mg of |                     |                     |                     | Dry weight per<br>cent of wet weight<br>Liver |
|------------------------------|---|--------------------------------|----------------------------------|---------------------|---------------------|---------------------|-----------------------------------------------|
|                              |   |                                | thymus                           | spleen              | heart               | kidneys             |                                               |
| 0                            | 5 | 29.7 <sup>±</sup> 1.4          | 76 <sup>±</sup> 19               | 169 <sup>±</sup> 53 | 130 <sup>±</sup> 18 | 447 <sup>±</sup> 63 | 31.4 <sup>±</sup> 1.1                         |
| 12                           | 4 | 28.9 <sup>±</sup> 1.3          | 65 <sup>±</sup> 19               | 131 <sup>±</sup> 23 | 150 <sup>±</sup> 34 | 395 <sup>±</sup> 55 | 31.4 <sup>±</sup> 2.0                         |
| 24                           | 4 | 30.1 <sup>±</sup> 1.0          | 53 <sup>±</sup> 17               | 128 <sup>±</sup> 7  | 126 <sup>±</sup> 19 | 342 <sup>±</sup> 22 | 31.0 <sup>±</sup> 0.3                         |
| 36                           | 4 | 29.1 <sup>±</sup> 1.0          | 48 <sup>±</sup> 13               | 118 <sup>±</sup> 31 | 120 <sup>±</sup> 7  | 398 <sup>±</sup> 29 | 30.1 <sup>±</sup> 1.8                         |
| 48                           | 4 | 29.8 <sup>±</sup> 1.3          | 58 <sup>±</sup> 10               | 138 <sup>±</sup> 10 | 119 <sup>±</sup> 10 | 412 <sup>±</sup> 28 | 30.5 <sup>±</sup> 1.0                         |
| 72                           | 4 | 28.8 <sup>±</sup> 0.7          | 54 <sup>±</sup> 26               | 119 <sup>±</sup> 18 | 111 <sup>±</sup> 6  | 368 <sup>±</sup> 33 | 31.7 <sup>±</sup> 1.6                         |

<sup>x</sup>Mean  $\pm$  S.E.



Fig. 1

Body weight, per cent of morning weight

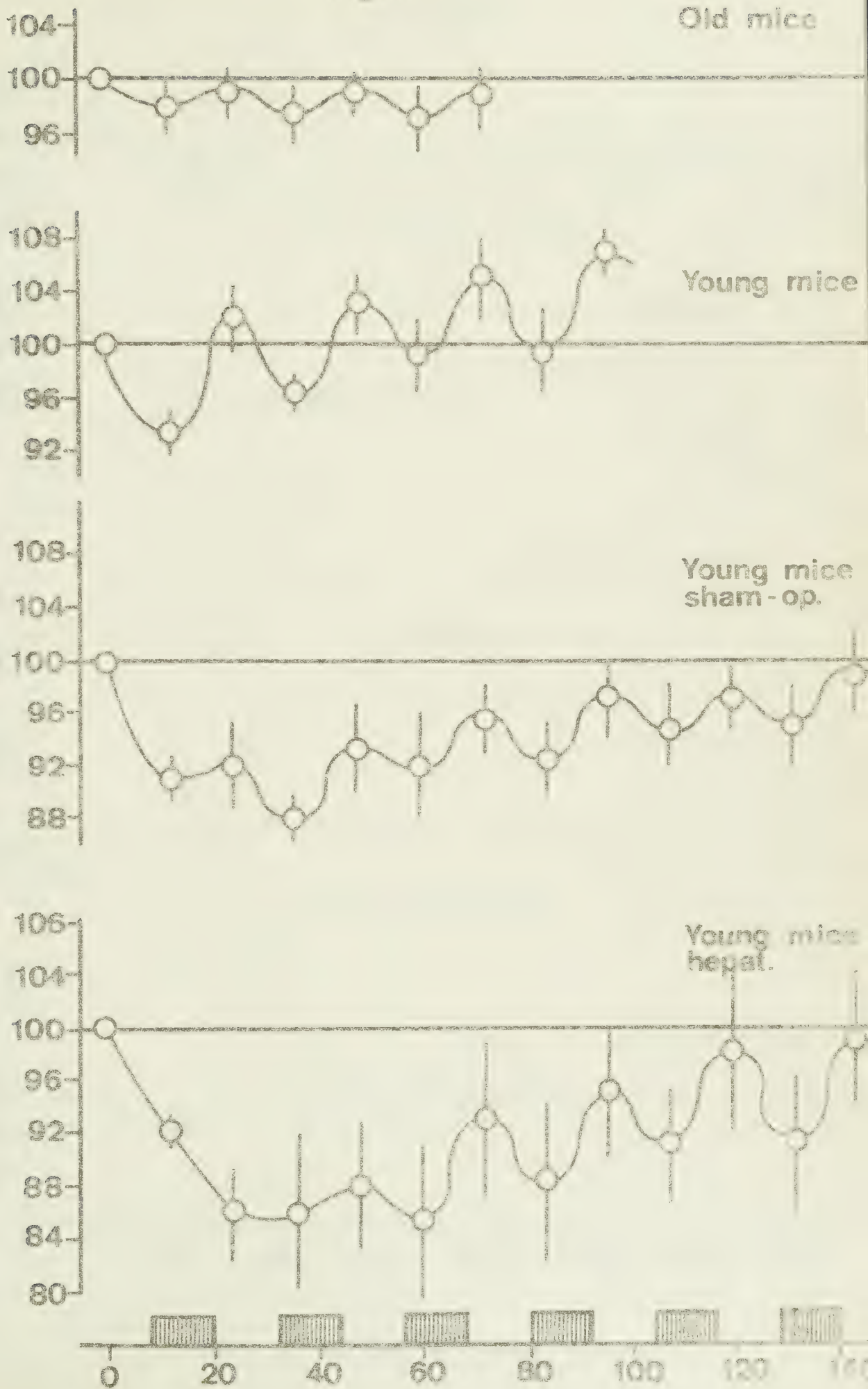




Fig. 2A

Weight, per cent of normal

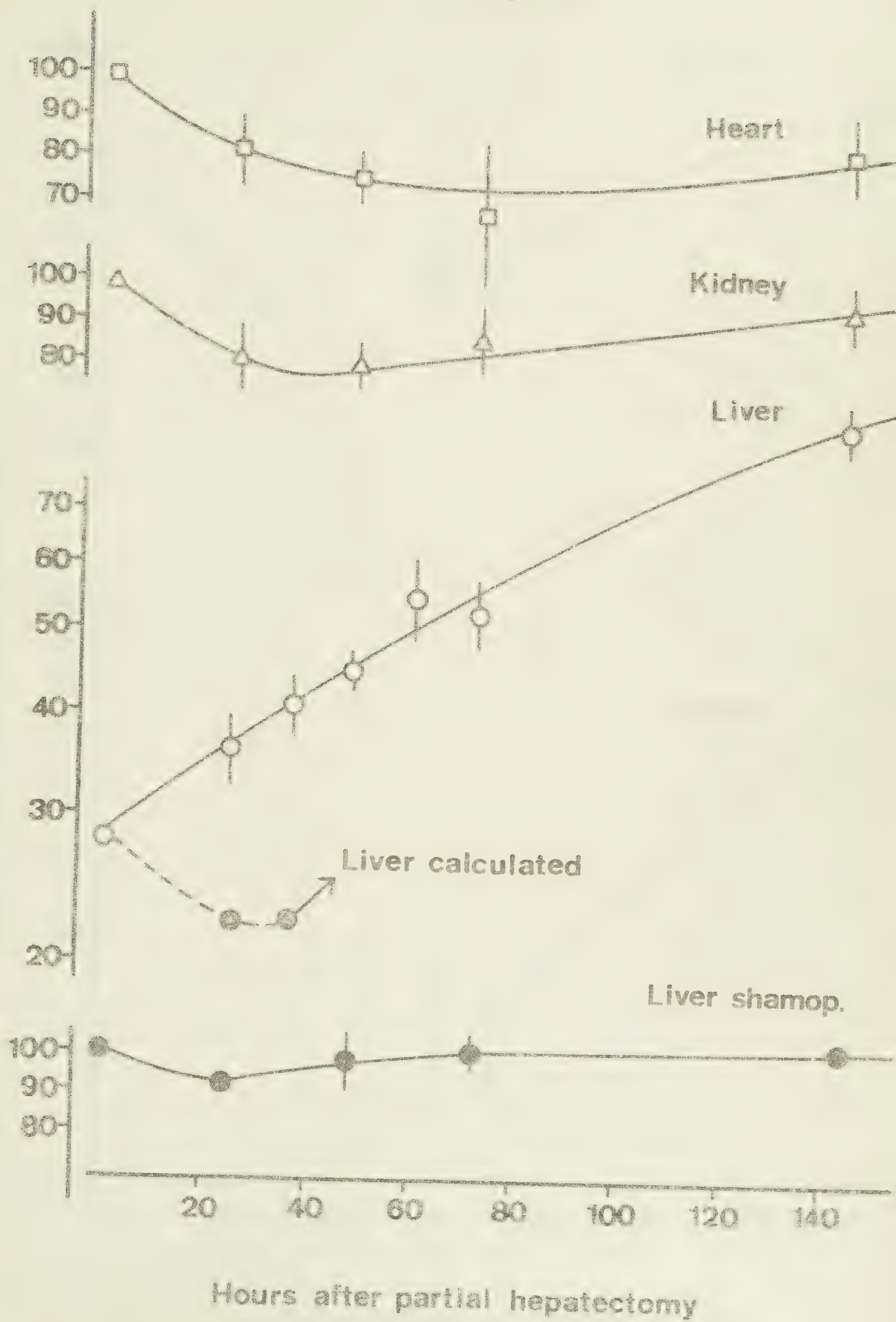






Fig. 2B

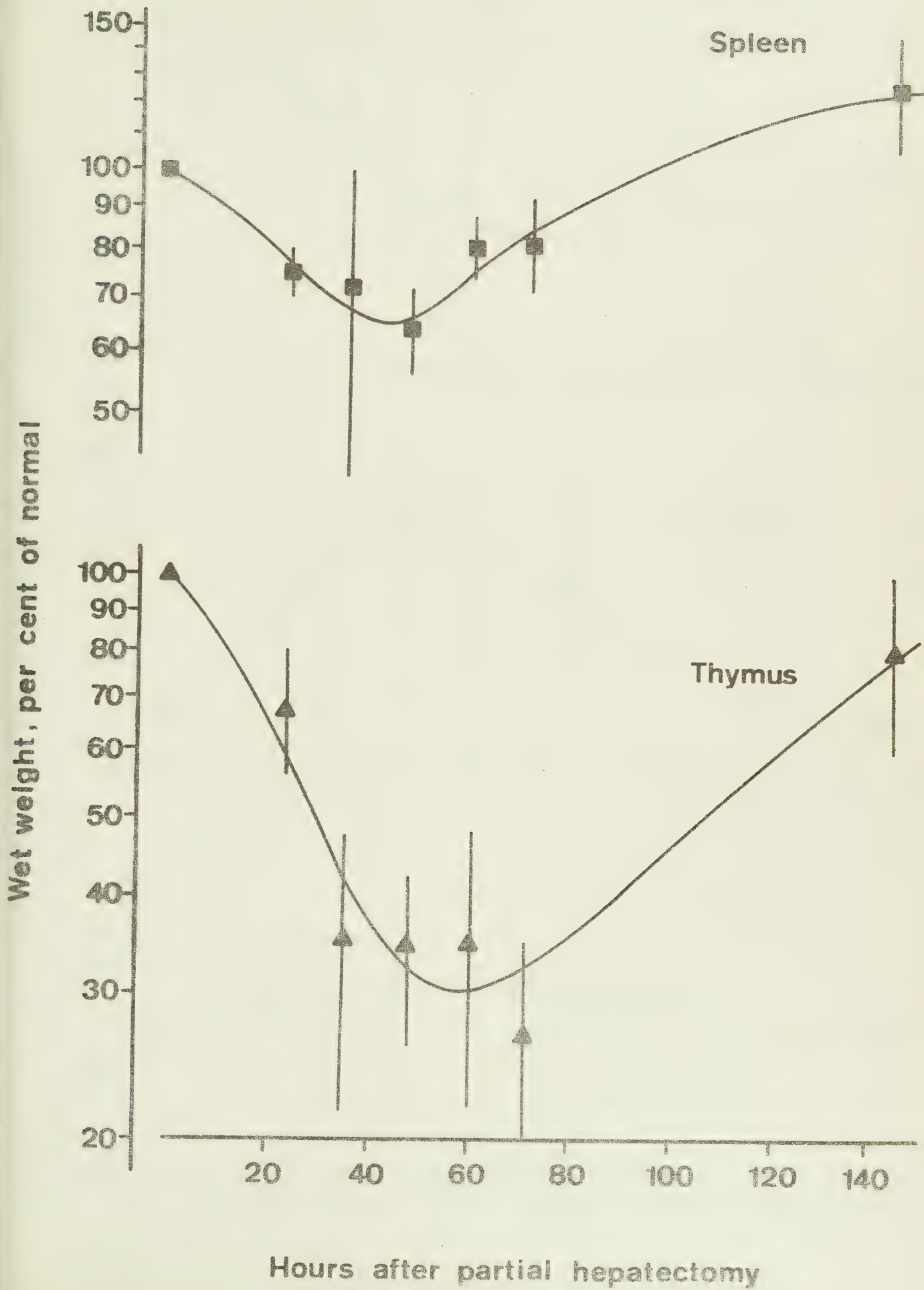
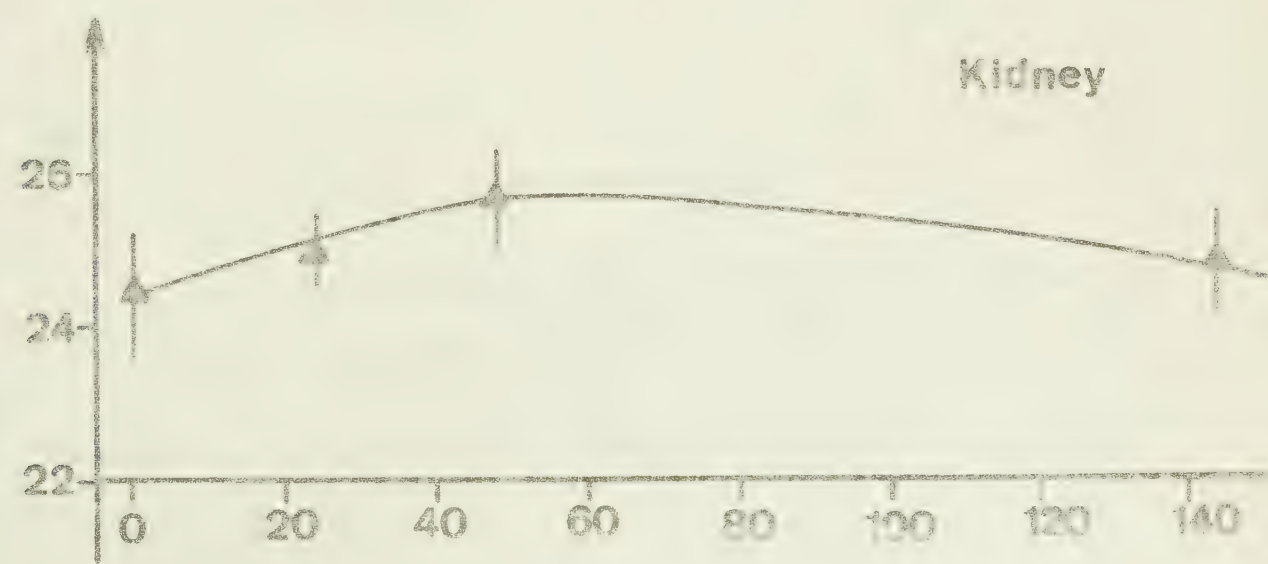
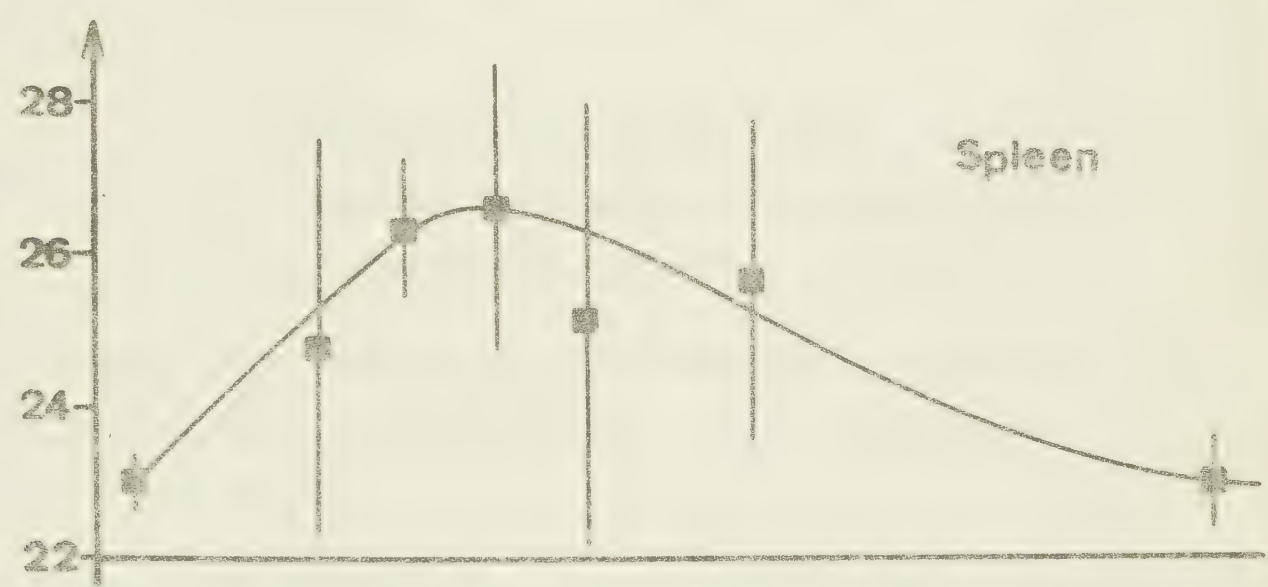
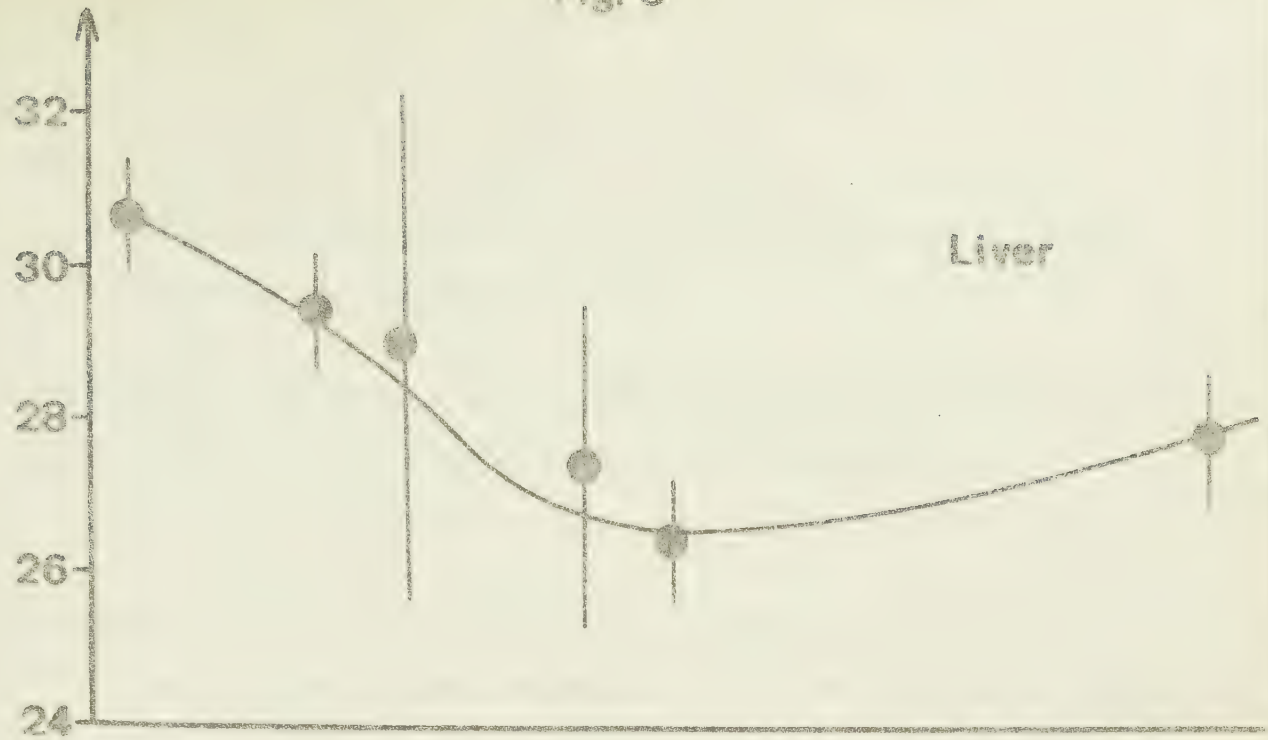




Fig. 3

Dry matter content, per cent of wet weight



Hours after partial hepatectomy



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## Protein, Nucleic Acids and Cell Structure in the Regenerating Mouse Liver\*

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**Summary.** Prior to the onset of mitotic activity in the regenerating mouse liver, the concentrations of total protein and DNA, but not of RNA, decreased to 93 per cent of their levels in normal liver. During maximum mitosis (48-72 hours), the DNA and RNA concentrations were 110 per cent of their normal levels. The concentrations of liver nucleic acids 24 hours after a sham-operation were also about 110 per cent of normal values.

Increased concentrations of insoluble protein were found at 12, 24 and 48 hours after partial hepatectomy. This may reflect an increase in the stability of the liver cell membranes during the regenerative period. Increased amounts of various cytoplasmic membranes were indicated by electronmicroscopy and may also have contributed to the increase in insoluble protein.

A temporary increase in the measured solubility of the liver protein occurred during maximum mitosis. It is suggested that this was due to the association of protein with lipids during the depletion of accumulated fat from the regenerating liver.

**Key words:** Liver regeneration -- Membranes -- Nucleic acids -- Proteins -- Solubility -- Biochemical analysis and Electronmicroscopy.

### Introduction

During liver regeneration after partial hepatectomy, unbalanced growth occurs, with cell enlargement and fat infiltration preceding DNA synthesis and mitotic activity (Bucher, 1963, 1967; Bengtmark, 1970; Grundman and Seidel, 1969). Although histological changes have been observed during the regenerative process (Jordan, 1964; Trotter, 1964; Bartok, Totovic, and Gedigk, 1967), little attention has been paid to the influence of such changes upon the results of biochemical analysis. There are indications that the state of the cell membranes is altered in the liver of partially hepatectomized mice (Lewan, 1971), and this may affect the solubility of the liver protein. To determine whether this is so, the concentrations of soluble and insoluble protein have been registered in mouse liver during regeneration and related to the amounts of various membranous structures in the liver cells, as apparent in ultrathin sections. Since there are large fluctuations in the composition of the regenerating liver (Tsuboi *et al.*, 1954; Van Lancker and Sempoux, 1958), the concentrations of nucleic acids have also been determined. The results have been compared to those found 24 hours after a sham-operation.

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In addition, the concentrations of total protein in the livers of hepatectomized mice have been compared with those found in the serum, the thymus and the spleen during the regenerative process and 24 hours after a sham-operation.

### Materials and Methods

*Experimental Animals.* Sixty-seven young male Swiss albino mice, weighing 28–33 g, from Anticimex, Uppsala, were used for the biochemical experiments. Eight young female C3H mice, weighing 16–17 g, from an inbred strain kept at the Institute, were used for electron microscopic examination. All animals were kept under standard conditions of light and temperature and were fed a standard laboratory diet ad libitum before and after the operation.

*Partial Hepatectomy.* Partial hepatectomy was carried out essentially according to Higgins and Andersson (1931), as described by Heby and Lewan (1971). The amount of liver removed at the operation in a control group of ten Swiss albino mice constituted  $68.4 \pm 2.0$  per cent of the total liver weight. In C3H mice the corresponding figure was  $72.3 \pm 2.0$  per cent (Heby and Lewan, 1971).

In sham-operated animals the peritoneum was opened and the liver lobes handled.

*Autopsy and Tissue Treatment.* The mice to be used for biochemical analysis were decapitated in groups of ten at 12, 24, 48 and 72 hours after partial hepatectomy; seven mice were decapitated 144 hours after the operation. Ten sham-operated mice were decapitated 24 hours after operation. The organs to be analyzed were quickly removed, weighed and homogenized in ten volumes of ice cold phosphate-saline Luffer (0.145 M NaCl, 0.01 M sodium phosphate pH 7.1) with ten strokes of a motor-driven glass-teflon homogenizer.

For electron microscopic examinations, samples were taken from two ~~normal~~ mice and 6 hepatectomized mice killed at 13, 20, 43, 70, 100 and 140 hours after the operation. normal

*Weight Registrations.* The weight of the regenerating liver at autopsy was expressed as a percentage of the liver weight at operation. The latter was calculated from the weight of the resected lobes, on the assumption that these constituted 68.4 per cent of the total liver mass (Swiss albino mice).

The weights of the thymus and the spleen of operated animals at autopsy were expressed as percentages of the mean weights of the thymus ( $80 \pm 5$ ) and the spleen ( $10 \pm 2$ ) of unoperated control mice.

*Analysis of Nucleic Acids.* The amounts of nucleic acids per gram of liver were estimated in samples of liver homogenate according to Munro and Fleck (1958), as described elsewhere (Heby and Lewan, 1971).

*Analysis of Protein.* Protein determinations were performed according to Lowry (1951), with Bovine Serum Albumin, fraction V (Sigma) as the standard. The values were expressed per gram wet weight of the organs analyzed. Total protein was estimated in samples of the homogenate. Soluble protein was estimated in the supernatant after centrifugation of the homogenate at 3000 g for 45 min. Insoluble protein was calculated as the difference between total and soluble protein.

*Electronmicroscopy.* Small pieces of liver were fixed in  $\text{KMnO}_4$  for 1 hour at  $+4^\circ\text{C}$  (Luft, 1956), embedded in Vestopal W and sectioned on an LKB ultratome. They were examined in a Hitachi HS-7C electronmicroscope. h

### Observations

*Organ Weights (Fig. 1).* The wet weight of the liver remnant increased during the period investigated to 80 per cent of the total liver weight at operation.

The weight of the thymus decreased to 40 per cent of that in unoperated animals, with a tendency towards weight restoration at 144 hours after the operation. The weight of the spleen decreased to 80 per cent and then increased to 140 per cent of the spleen weight in unoperated animals.

*Nucleic Acids (Fig. 2).* The RNA concentration in the liver rose slowly during regeneration to around 110 per cent of the normal value, which was  $7.3 \pm 0.3 \text{ mg/g}$ .



per gram of liver. In sham-operated animals the RNA concentration increased more rapidly than in regenerating liver.

There was a 7 per cent decrease in the DNA concentration during the first 24 hours of liver regeneration, followed by an increase to 112 per cent of the normal value, which was  $2.2 \pm 0.04$  mg per gram of liver. In sham-operated animals the DNA concentration increased to 115 per cent of the normal value.

*Protein (Figs. 3, 4).* There was a 7 per cent decrease in the total amount of protein per gram of liver during the period of investigation and a tendency to return to the normal value by the end of that time (Fig. 3). Except at 48 and 72 hours, a larger part than in normal liver was constituted by insoluble protein. In the thymus, a 10 per cent decrease in the amount of total protein per gram wet weight occurred during liver regeneration (Fig. 4). There was no change in the amount of total protein per gram wet weight in the spleen until 72 hours after the hepatectomy (Fig. 4). At the end of the regenerative period, however, the concentration of total protein in the spleen was 8 per cent lower than in unoperated mice.

An eleven per cent decrease in the protein concentration of the blood serum was observed during the first 48 hours of liver regeneration, followed by a return towards normal values (Fig. 4).

In all organs analyzed, the protein concentration tended to increase after sham-operation, this being quite contrary to the trend observed after hepatectomy (Figs. 3, 4).

*Electron Microscopy (Fig. 5).* Increased amounts of various cytoplasmic membranous structures were seen in the regenerating liver at all stages analyzed.

#### Discussion

There are obviously large variations in the extractability of liver protein at different intervals after hepatectomy, as well as in the concentrations of total protein, DNA and RNA. In the period prior to the onset of mitotic activity, which occurs between 48 and 72 hours after hepatectomy (Heby and Lewan, 1971), these changes generally pursue the opposite course to those seen in sham-operated animals.

The concentration of nucleic acids in the liver increased after sham-operation. Since there was no change in water content (Lewan, unpublished observations), the increase probably reflects a depletion of stored products. This may have occurred during the period of light starvation associated with sham-operation (Enwonwu *et al.*, 1971). Release of protein is indicated by the thirty per cent increase in serum protein concentration following sham-operation.

The immediate response of the liver to partial hepatectomy differed from that to sham-operation. It is true that, even in hepatectomized mice, the liver is depleted of stored products because of starvation (Jordan, 1964; Treloar, 1964). There is, however, a considerable accumulation of fat during the first 24 to 48 hours after hepatectomy (Tsuboi *et al.*, 1954). This explains the observed decrease in DNA concentration. When the accumulated products disappear, between 48 and 72 hours after operation, the DNA concentration reaches higher values comparable with those found 24 hours after a sham-operation (Fig. 2). Despite the diluting effect of fat accumulation, there was no decrease in RNA concentration during





early regeneration. This must be because synthesis of RNA increases very soon after operation (Bucher and Swaffield, 1963). The decrease in protein concentration prior to mitosis was of the same order of magnitude as the decrease in DNA concentration, despite reports that increased protein synthesis precedes elevated DNA synthesis (Majumdar *et al.*, 1967; Grisham, 1962). The slow return of the protein concentration towards the normal level indicates an increase in protein transport from the liver. However, despite increased protein synthesis and transport, the liver remnant is unable to maintain the level of serum protein, as found in this study (Fig. 4) and in previous investigations on the rat (Morgan and Brackenridge, 1962; Schreiber *et al.*, 1970).

High concentrations of protein were found in the thymus, spleen and blood serum after sham-operation, but not after partial hepatectomy, indicating a general protein deficiency in the hepatectomized animals. The changes in total protein concentrations in the thymus and spleen are associated with rapid weight changes in these organs (Fig. 1) and may be influenced by changed proportions between the cell populations within them during involution and growth (Leoughlin, 1962).

Increased amounts of various membranous structures were observed in the cytoplasm of liver cells at all stages of regeneration analyzed. Differences in the intralobular localization of these structures might explain these observations (Becker and Lane, 1968). However, increases in the synthesis (Lardetaky *et al.*, 1956) and concentrations (Lengmark, 1969) of phospholipids have been observed in regenerating liver. An increased stability of the cell membranes at homogenization was found in a previous study of regenerating mouse liver (Lewin, 1971). The increase in the amount of membranous structures and the greater stability of the membranes together result in larger cell fragments being obtained at homogenization. Since large cell fragments sediment more readily than small ones during centrifugation, a larger proportion of tissue protein might be recovered in the insoluble protein fraction from regenerating liver than in that from normal liver. Decreased concentrations of insoluble protein were, in fact, found at 12, 24 and 48 hours after operation. There was, however, a temporary increase in the measured concentration of insoluble protein during maximum mitosis.

A decrease in the amount of insoluble protein may be interpreted as a differentiation, but a retention of the differentiated state during mitosis has been suggested (Becker and Lane, 1968) and is supported by the electron micrographs in this study. Some fat was recovered in the soluble protein fraction at centrifugation, and the liver is depleted of fat between 18 and 72 hours after operation (Tsuboi *et al.*, 1954; Trotter, 1964). fat-associated protein may have contributed to the temporary increase in soluble protein at this time. Proteinaceous membranous structures associated with fat (Laloum, 1964) as well as the protein moiety of lipoprotein (Lengmark, 1969; Husakova and Simch, 1970) may be of importance for the depletion of the fat store by metabolism or transport.

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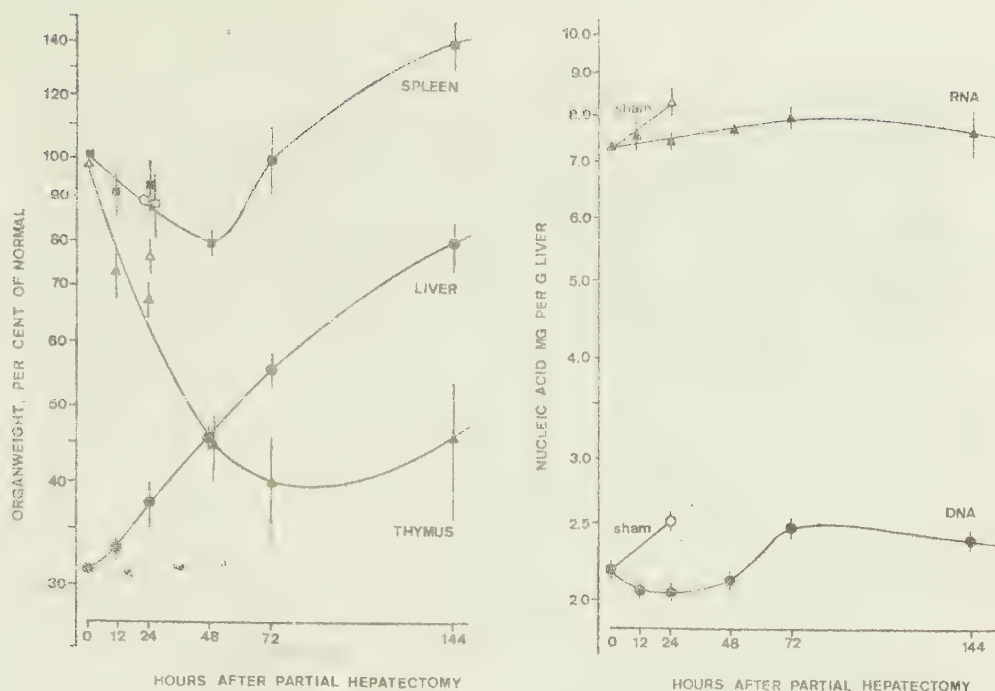


Fig. 1. Organ weights after operation, expressed as percentages of the organ weight in normal animals. Semilogarithmic scale. Mean  $\pm$  S.E. Open symbols represent sham-operated animals

Fig. 2. Concentrations of nucleic acids in mg per gram wet weight after operation. Semi-logarithmic scale. Mean  $\pm$  S.E.

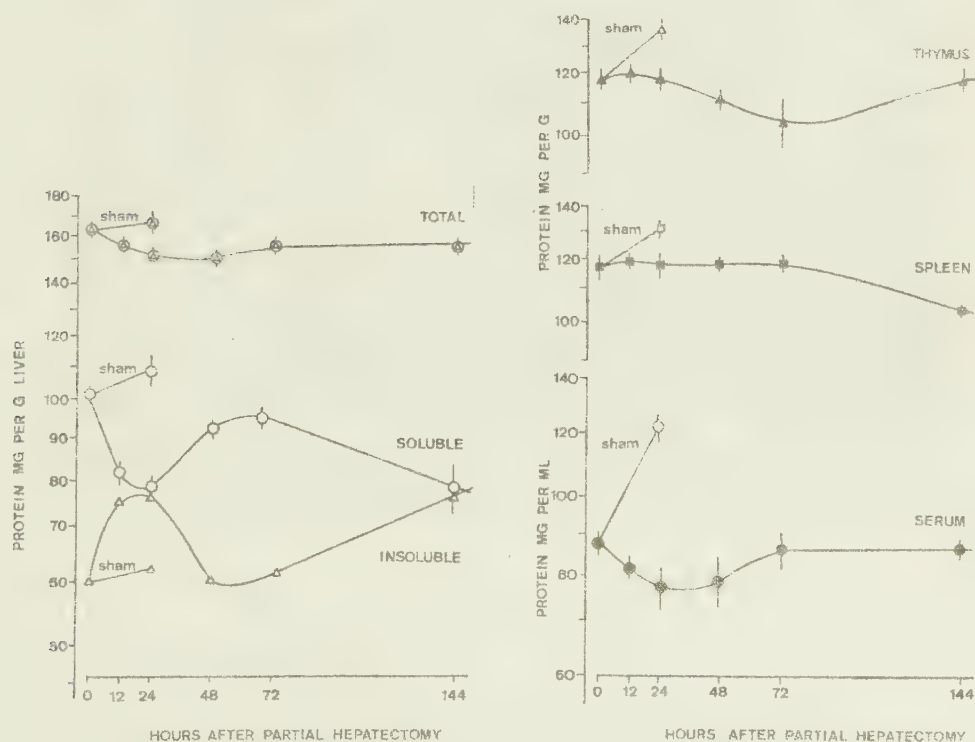


Fig. 3. Concentrations of liver proteins after operation in mg per gram wet weight. Semi-logarithmic scale. Mean  $\pm$  S.E.

Fig. 4. Concentrations of total protein after operation in thymus and spleen (mg per gram wet weight) and serum (mg per ml). Semi-logarithmic scale. Mean  $\pm$  S.E.





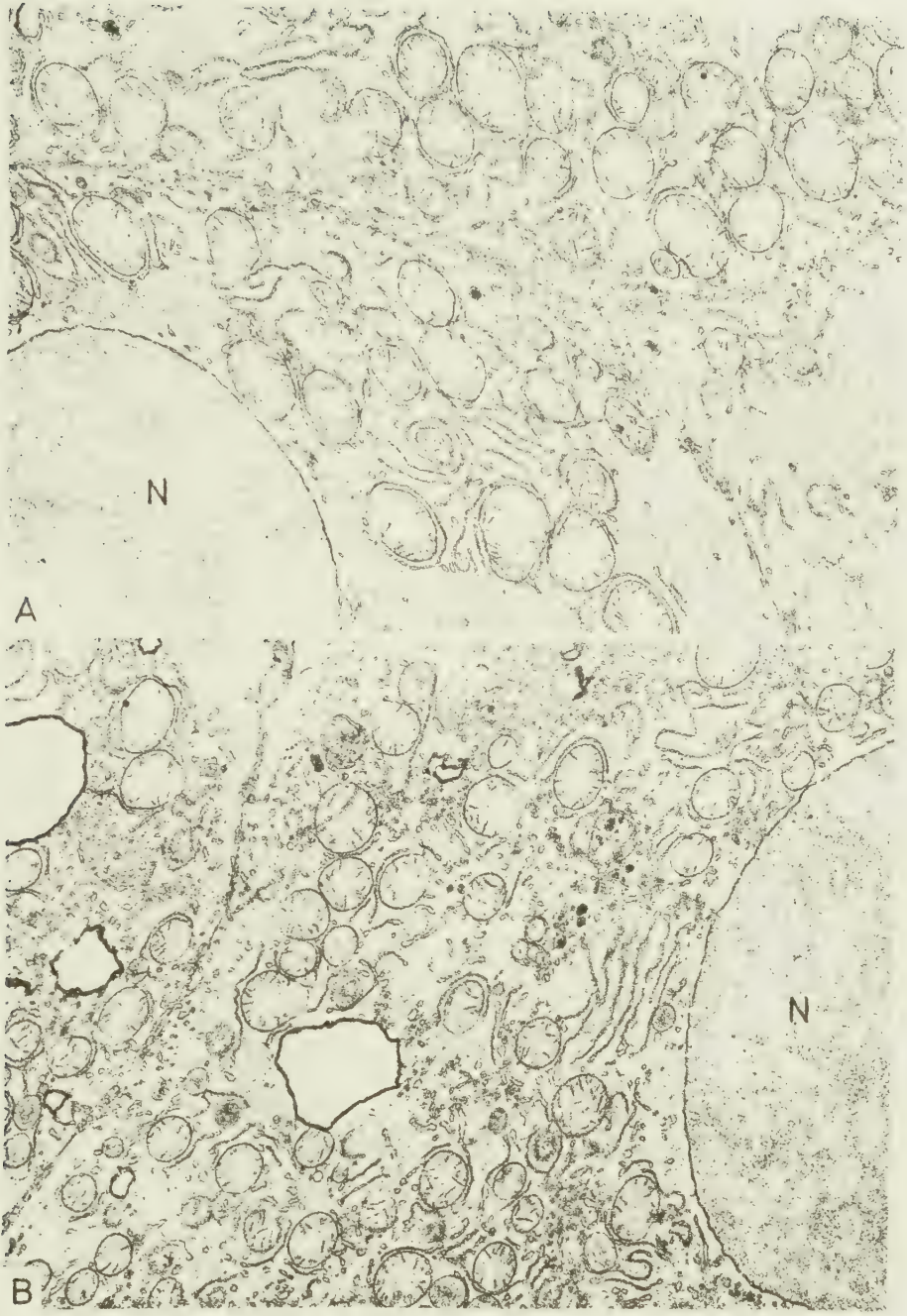
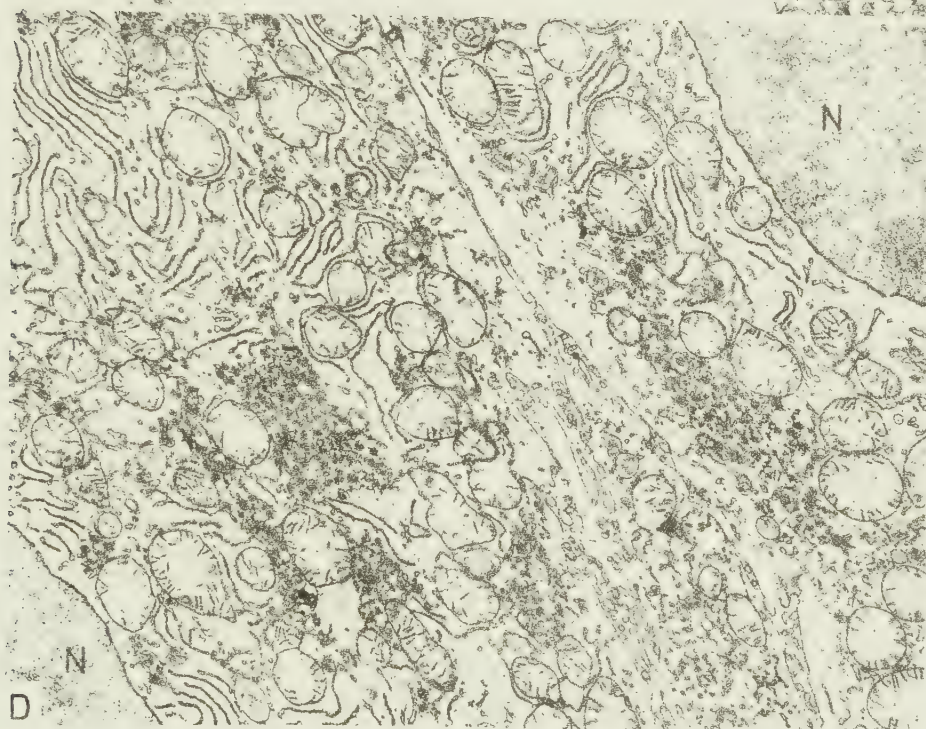
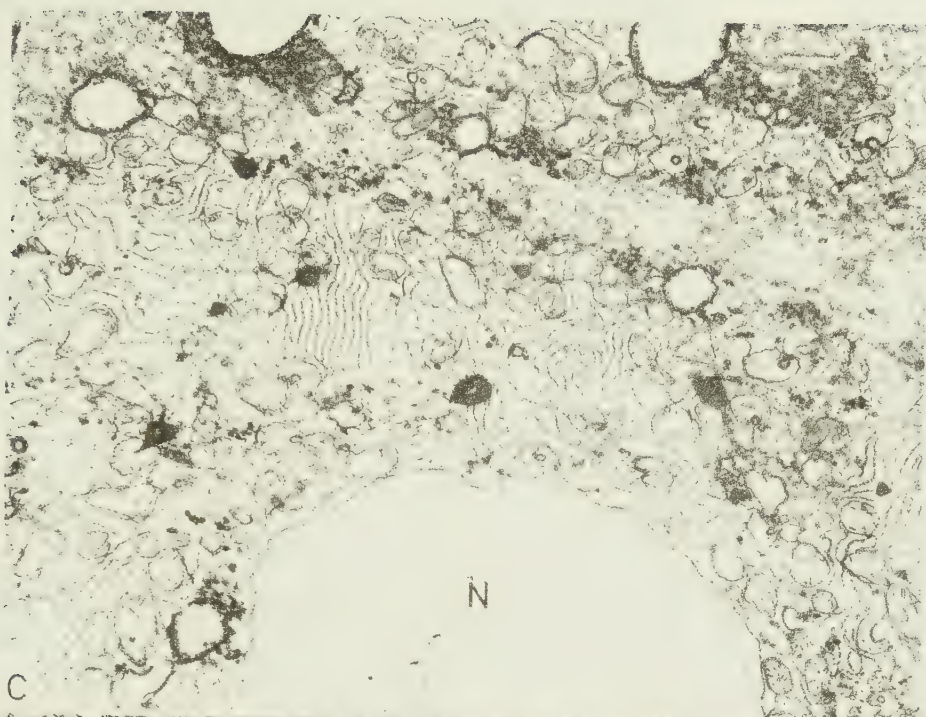


Fig. 5 A-D. Electron micrographs of  $\text{KMnO}_4$ -fixed liver. Magnification 21600. A normal liver. B Regenerating liver, 20 hours after partial hepatectomy. C Regenerating liver, 43 hours after partial hepatectomy. D Regenerating liver, 140 hours after partial hepatectomy.













## Putrescine and Polyamines in Relation to Nucleic Acids in Mouse Liver after Partial Hepatectomy\*

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*Summary.* Changes in polyamine and nucleic acid concentrations were studied during the regeneration of mouse liver. Partial hepatectomy caused an early accumulation of putrescine. There was a rapid increase in spermidine concentration parallel to an increase in RNA concentration and concomitant with a decrease in spermine concentration. Only moderate changes in DNA concentration were found during the growth period. The results are discussed in relation to transcription and replication during the restoration of mouse liver after partial hepatectomy.

### Introduction

The polyamines are aliphatic, nonprotein, nitrogenous bases, widely distributed in nature. They occur in animals, plants and a variety of micro-organisms (Tabor and Tabor, 1964). In the present report spermine,  $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$ , spermidine,  $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$ , and putrescine,  $\text{NH}_2(\text{CH}_2)_4\text{NH}_2$ , will be referred to as polyamines although strictly speaking putrescine is a diamine.

Results obtained from calf thymus nuclei and rat liver nuclei, isolated by a non-aqueous technique, showed that the nuclear and cytoplasmic concentrations of spermidine and spermine do not differ appreciably (Stevens, 1966). In mammalian tissues, there is generally more spermidine present than spermine (Rosenthal and Tabor, 1956).

Although we now have a wider knowledge of the distribution, metabolism and biosynthesis of the polyamines, their effects on biological systems are still little known. There is, however, evidence that these amines are concerned with the metabolism and function of the nucleic acids (Stevens, 1970). In vitro, the polyamines interact strongly with DNA and RNA (Heby and Agrell, 1971). In agreement, many recent observations suggest a function for the tissue polyamines as physiological stabilizers of nucleic acids. Furthermore, they affect enzyme reactions involving the synthesis of nucleic acids, particularly RNA synthesis.

In animal tissues several lines of evidence have indicated an involvement of polyamines in rapid tissue growth. The regenerating liver has been used advantageously to study changes during rapid growth and the proliferation of cells. Surgical removal of 70 per cent of the hepatic parenchyma of the mouse provokes compensatory hypertrophy and hyperplasia in the residual lobes. Almost complete restoration of the original tissue mass is achieved within 8 to 10 days (Yokoyama *et al.*, 1953).



Numerous investigators have studied the biochemical events that occur after hepatectomy in order to elucidate which changes regulate tissue growth (Bucher, 1963; 1967; Bengmark, 1970). Studies of changes in the polyamine content of the liver remnant after hepatic resection have been made only in rats (Dykatra and Herbst, 1965; Raina *et al.*, 1966).

The present study was undertaken in order to investigate possible polyamine variations in regenerating mouse liver and their relation to growth, mitosis and nucleic acids.

### Material and Methods

*Experimental Animals.* Young, female, C3H mice from an inbred strain kept at our Institute, were used for the experiments. They were given standard laboratory feed and allowed to eat and drink ad libitum. It is known that age is an important factor affecting the amounts of tissue polyamines (Jänne *et al.*, 1964; Shimizu *et al.*, 1965). The process of liver regeneration is also affected by the age of the animal, in that rapidly growing young rats restore tissue mass and the cell population more rapidly than do adults (Bucher, 1967). For these reasons all animals chosen were between 5 and 6 weeks old. Variations in bodyweight were kept within narrow limits, never exceeding  $\pm 5$  per cent of the mean value (16.2 g).

*Partial Hepatectomy.* Partial hepatectomy, essentially according to Higgins and Anderson (1931), was carried out with the animals lightly anaesthetized with pentobarbitone (Nembutal, Abbot Laboratories).

Since the number of mitoses in the regenerating rat liver exhibits considerable diurnal variation (Jaffe, 1954), we always carried out hepatectomy at approximately the same time of day, viz. between 9<sup>00</sup> and 12<sup>00</sup> a.m. The amount of the liver removed from a group of ten mice constituted  $72.3 \pm 2.0$  per cent of the total liver weight, a value which is somewhat larger than that earlier reported (Tsuboi *et al.*, 1954; Jardetzky *et al.*, 1956). This may be a consequence of a systematic difference in resection technique or of the strain, sex or state of the animals.

*Autopsy and Tissue Treatment.* The mice were decapitated in groups of three at 13, 20, 30, 40, 43, 46, 50, 60, 70, 80, 100 and 140 hours after operation. No significant variations were found in the quantities of liver polyamines or nucleic acids after laparotomy or anaesthesia, and thus ten unoperated mice were used to obtain the normal values. The livers were quickly removed, weighed and chilled. Samples of liver tissue were removed from each mouse for determination of the mitotic index. The livers were kept at  $-25^{\circ}\text{C}$  until the time of analysis, when they were separately homogenized in saline in a teflon homogenizer (one part of tissue to nine parts of 0.9% NaCl). All procedures in tissue treatment were performed below  $+4^{\circ}\text{C}$ . A sample from each homogenate was taken for DNA and RNA assay, and the rest was used for polyamine analysis.

*Liver Growth.* The normal liver weights of the operated animals were calculated from the weights of the resected pieces on the assumption that these constituted 72.3 per cent of the total liver mass. The weight of the regenerating liver at sacrifice was expressed as a percentage of the normal liver weight.

*Mitotic Index.* To determine mitotic indices small slices of each liver were fixed in absolute ethanol:glacial acetic acid, 3:1. The fixed pieces were embedded in paraffin, cut in 5  $\mu$  sections and stained with haematoxylin and eosin. After observing 2000 to 20000 parenchymal nuclei, depending upon the mitotic activity, the mitotic index was determined as the number of mitoses per 100 nuclei.

*Preparation and Analysis of Polyamines.* The polyamines were extracted from the homogenates as follows: Equal volumes (5 ml) of liver homogenate and cold 10% trichloroacetic acid were mixed in order to deproteinize the tissue sample. The precipitate was removed by centrifugation, the supernatant was extracted three times with an equal volume of diethyl ether to remove the acid, and the ether remaining with the aqueous extract was removed by bubbling nitrogen through the solution. The amines in the sample were separated from nonbasic substances by the n-butanol extraction method of Raina (1963). The n-butanol layer was then acidified and freeze dried. The residue was dissolved in a small volume of 0.1 M



HCl (0.1 mg EDTA/ml) and stored in a small testtube until analysis. The final separation of the polyamines was performed by thin-layer chromatography using a method described by Hammond and Herbst (1968). The staining of polyamines with ninhydrin and elution of the stained fractions for quantitative determination have previously been described (Heby and Agrell, 1971).

*Preparation and Analysis of Nucleic Acids.* The samples for DNA and RNA estimations were prepared according to the Schmidt and Tannhauser method as modified by Munro and Fleck (1966) omitting the lipid extraction. RNA was estimated by UV-absorption on the assumption that 32  $\mu\text{g/ml}$  gives an extinction of 1.000 at 260 m $\mu$ , as worked out for rat liver (Munro and Fleck, 1966). DNA was estimated by the diphenylamine reaction (Burton, 1956).

*Chemicals.* Putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride, used as standards, were commercial analytical grade preparations obtained from Calbiochem. Sigma calf thymus DNA (type V) was used as standard. The reagents and solvents used were of analytical grade, mainly manufactured by BDH.

### Results

Regenerative growth after partial hepatectomy measured as increase in liver wet weight, is illustrated in Fig. 1. The growth rate of the regenerating mouse liver is very slow during the first day, but from then on there is a marked increase, which is most pronounced around 50 hours after operation. At the end of the 140 hours experimental period, 75 per cent of the pre-operative liver mass is restored.

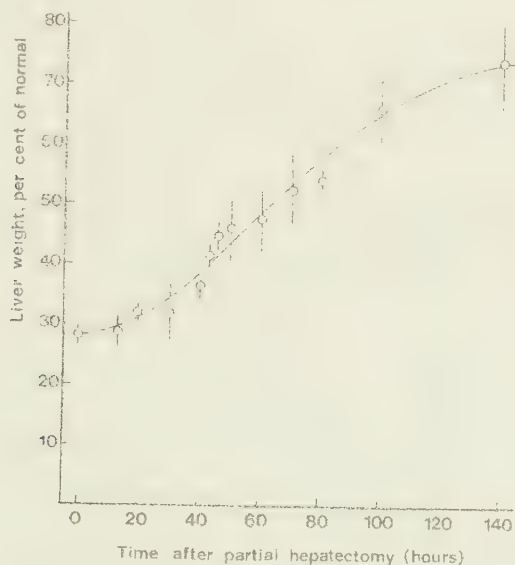


Fig. 1. Changes in wet liver weight, expressed in per cent of normal weight, following partial hepatectomy in the mouse. Mean  $\pm$  S. D.

The estimated number of parenchymal nuclei in mitosis is presented in Fig. 2. Few mitoses were noted prior to 20 hours. Thereafter the number of mitoses rapidly increased to a peak between 40 and 50 hours after hepatectomy, when about 3.7 per cent of the nuclei were in mitosis. Following this, the number of





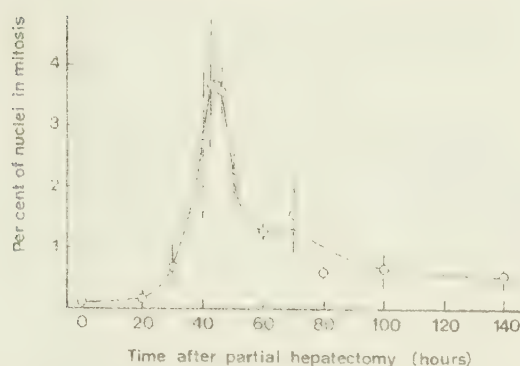


Fig. 2. Percentage of parenchymal nuclei in mitosis during mouse liver regeneration. Mean  $\pm$  S. D.

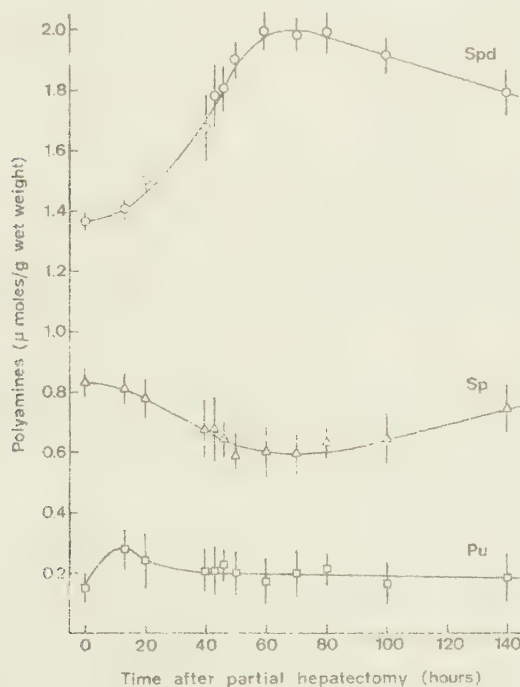


Fig. 3. Concentrations of polyamines during mouse liver regeneration. *Pu* putrescine, *Spd* spermidine and *Sp* spermine. Mean  $\pm$  S. D.

mitoses rapidly decreased, although it did not reach the control level during the experimental period. In Fig. 2 there are indications of a second mitotic peak appearing on the morning of the fourth day.

The process of mouse liver regeneration in the present study, described by means of changes in liver mass and mitotic index, is mainly in accordance with previous reports (Yokoyama *et al.*, 1953; Wilson *et al.*, 1953). Minor differences in



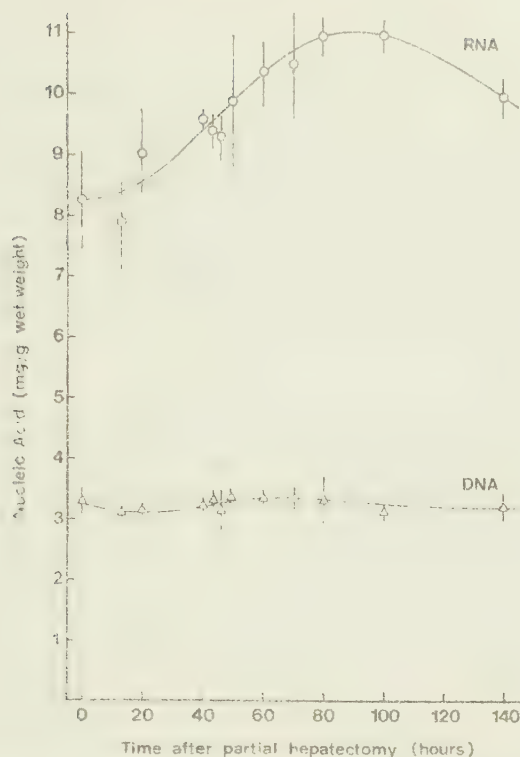


Fig. 4. Concentrations of nucleic acids during mouse liver regeneration. Mean  $\pm$  S. D.

the results may be due to such differences as time of operation, anaesthesia, and the strain and sex of mice used. The regenerative rate of rat liver is related to the mass of the resected portion (Weinbren and Taghizadeh, 1965) and is faster in young animals than in older ones (Bucher, 1963) and this may also hold true for mice.

Changes in the mean concentrations of the polyamines after partial hepatectomy are presented in Fig. 3. The first change noted was in the putrescine concentration, which was markedly increased as early as 13 hours after operation, reaching 187 per cent of the normal value. A very marked change was also found in the spermidine concentration, which was clearly increased at 20 hours and reached a peak value of 146 per cent of the normal value at 70 hours. In contrast to the increase of the putrescine and the spermidine concentration, that of spermine decreased towards a minimum of 71 per cent of the normal value at 70 hours. With declining liver growth, at the end of the experimental period, the polyamine concentrations approached normal values. The quantitative polyamine changes in the mouse liver after partial hepatectomy are comparable with those in the rat liver, after a corresponding resection (Dykstra and Herbst, 1965; Raina *et al.*, 1966).

Changes in the mean concentrations of the nucleic acids after partial hepatectomy are presented in Fig. 4. The most marked changes were seen in the RNA



concentration, which increased towards a peak value of 133 per cent of the normal value around 90 hours after operation. The DNA concentration was rather constant during the regeneration but showed a slight decrease during the first day. The quantitative DNA changes are essentially the same as those reported earlier by Jardetzky *et al.*, (1956) and the RNA changes are comparable with those reported (Tsuboi *et al.*, 1954), although more pronounced.

### Discussion

Following partial hepatectomy, the residual liver changes its normal metabolism in a characteristic way, which leads to early cell enlargement and new relations between the cellular components. During the first day, there is an increase in RNA synthesis, heavy fat infiltration, glycogen depletion and a concomitant intracellular digestion caused by elevated lysosomal activity (Bucher, 1967). The early RNA synthesis is followed by an increase in protein synthesis (Rabes and Brändle, 1969) and, subsequently, by DNA synthesis, the latter being most intense around 33 hours in the mouse (Bade *et al.*, 1966).

During early regeneration the cells of the residual liver are modified so as to pass from a "G<sub>0</sub>" phase into the replicating and dividing phases of the cell cycle. The factors responsible for this modification are still unknown, but RNA and protein produced at the early gene activation are supposed to be of importance for DNA synthesis and mitosis (Baserga, 1968; Becker, 1969). The tendency for DNA to decrease in concentration during the pre-replicative period, Fig. 4, may be a result of the moderate growth at this time, Fig. 1. During the subsequent part of the experimental period, there is a close relationship between growth and DNA synthesis, reflected in the almost constant DNA concentration, Fig. 4.

Among the earliest changes after partial hepatectomy are those involved in polyamine metabolism. An early activation of putrescine and spermidine synthesis after operation is evident from Fig. 3. Putrescine, the product of ornithine decarboxylase activity, is known to be a precursor of both spermidine and spermine in rat liver (Siimes, 1967; Jänne, 1967). The biosynthesis of spermidine and spermine, respectively, involves the transfer of one, and two propylamine moieties from decarboxylated S-adenosylmethionine to a putrescine molecule. The increased accumulation of putrescine in the regenerating liver, Fig. 3, may be due to the sharp increase in the activity of ornithine decarboxylase which occurs during the first few hours (Russell and Snyder, 1968; Jänne and Raina, 1968). No other enzyme is known to undergo anywhere near so vast a change so early in the process of liver restoration (Bucher, 1963; 1967). As putrescine has been shown greatly to stimulate the S-adenosylmethionine decarboxylase system (Pegg and Williams-Ashman, 1968), the elevated putrescine level may be the main cause of the increased synthesis and accumulation of spermidine.

The pattern of spermidine accumulation very much resembles the RNA increment during the 140 hours experimental period, Figs. 3 and 4, which suggests some functional relationship between spermidine and RNA synthesis in regenerating mouse liver. A higher template activity for RNA synthesis was observed in regenerating liver chromatin, but there was no detectable difference in the ratio of histone to DNA, compared to that of normal liver chromatin (Bannai and



Terayama, 1969). Many authors have reported a stimulatory effect of polyamines on RNA synthesis (for references see Stevens, 1970). This effect of polyamines can be brought about mainly in two ways; either by interaction with nucleic acids (Abraham, 1968) or by competition with histones for nucleic acids (Agrell and Heby, 1971). The post-operative accumulation of RNA may partly depend on increased RNA synthesis and partly on increased stabilization of newly synthesized RNA molecules, favoured by the concomitant synthesis of polyamines. Polyamines have been found to inhibit RNA degradation, probably by stabilizing helical regions of RNA, thereby making it less susceptible to unfolding and digestion by ribonuclease. Molecular models to explain the double helix stabilization of DNA and RNA brought about by polyamines have been discussed in a recent paper (Heby and Agrell, 1971). Thus, the polyamines might contribute to the increased ribosome formation observed during liver regeneration (Chaudhuri and Lieberman, 1968) by promoting the association of ribosomal subunits and stabilizing the ribosomal structure. Many of the effects of polyamines on enzymes and ribosomes in vitro can be explained in terms of their interactions with the nucleic acids.

During the regeneration there is a marked decrease in the spermine concentration, but only a slight decrease in the DNA concentration, Figs. 3 and 4. The decreased spermine concentration may indicate a degradation of spermine to spermidine, a metabolic pathway evident from observations on regenerating rat liver (Sümes, 1967). As the spermine concentration decreases, the DNA may progressively become metabolically more active, e.g. due to labilization of its double-stranded structure. It is clear that the template activity of chromatin increases with removal of histones in vitro (Bannai and Terayama, 1969). Interactions between DNA and its associated proteins in vivo may be expected to change at the time of gene activation, and the net increase in polyamines, accounted for by spermidine, may partially displace histones from the chromatin, thereby increasing its template activity. This explanation is supported by the observation that putrescine and spermidine stimulate, whereas spermine inhibits, RNA synthesis in rat liver nucleolar preparations (Bain and Jänne, 1970). Putrescine also stimulates DNA synthesis if chromatin or nucleohistone are used as primers (Schwimmer, 1968).

The characteristic which most polyamine reactions have in common is that they involve nucleic acid interactions. Our findings lend further support to the hypothesis that polyamines are essential for the function of cells, rapidly growing cells in particular, probably as stabilizers of nucleic acids. Changes in the concentrations of polyamines may, together with acetylation and phosphorylation of histones, and changes in the ion composition, be of importance in regulating transcription and replication in rapidly growing tissues.

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The role played by the polyamines in various system of growth is further treated in a recent publication from this institute.

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Nuclear Proliferation and the Amount  
of DNA per Parenchymal Nucleus during Mouse  
Liver Regeneration\*

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*Summary.* When the number of parenchymal nuclei in regenerating mouse liver was estimated on the basis of nuclear counts in vestopal embedded sections, higher values were obtained than when the estimation was made on the basis of nuclear counts in homogenates. A selective fragmentation of small, newly formed nuclei is indicated, which may have contributed to an overestimation of the increasing polyploidy during mouse liver regeneration in earlier investigations. Studies on homogenates revealed that the stability of the cell membrane increased during the regeneration. This may have made it more difficult to obtain parenchymal cell nuclei in an unbroken state, free from cells, at homogenization.

The increase in the number of nuclei estimated in liver sections, during a period of 140 hours after partial hepatectomy, is in accordance with that indicated by the mitotic index, assuming that the mean mitotic duration is between one and two hours. Values in this range have actually been found in some preliminary experiments with colchicine. An initially high increase in the amount of DNA per nucleus indicates a long duration of the S and G<sub>2</sub> phases or possibly an accumulation in the liver of chromatin products from degraded tissues.

#### Introduction

The regenerating liver is often used in experimental studies of rapidly growing mammalian tissues (Bucher, 1963, 1967; Grundman and Seidel, 1969; Bengmark, 1970). After partial hepatectomy the cells of the residual liver enlarge (Harkness, 1957; Wilson *et al.*, 1953) and are infiltrated by fat (Bengmark, Olsson, and Svanberg, 1964; Trotter, 1964). After a lag period a vigorous DNA synthesis starts, followed by mitotic activity (Grisham, 1962; Barnum, Jardetzky, and Hallberg, 1957).

Because of the increase in liver weight (Brues, Drury, and Brues, 1936; Tsuboi *et al.*, 1954) during the lag period preceding DNA synthesis and mitotic activity, decreasing amounts of DNA and nuclei per gram of liver are to be expected during early regeneration. The fall in DNA concentration should be succeeded by a rise at the time of maximal DNA synthesis. At the time of maximal mitotic activity the number of nuclei per gram of liver tissue should also increase. When the regenerative process is completed the amount of DNA and nuclei per gram of liver tissue may be expected to approach normal values, unless an extensive increase in polyploidy occurs. Consequently, the amount of DNA per nucleus should rise when DNA synthesis starts, being maximal just before mitosis, and thereafter decrease towards normal values. A maximal value of 100–200% of the preoperative amount of DNA per nucleus may be expected, dependent upon the synchrony of the cells and the duration of the G<sub>2</sub> interval.



Only small variations have been reported in the DNA concentration of the regenerating liver (Tsuboi *et al.*, 1954; van Lancker and Sempoux, 1958). Large variations in the number of nuclei have been found, however, although the results of different authors are not entirely in agreement (Brues, Drury, and Brues, 1936; Price and Laird, 1950; Tsuboi *et al.*, 1954; van Lancker and Sempoux, 1958). Differences in age (Bucher and Glinos, 1950) and circadian rhythm (Echave Llanos *et al.*, 1971) of the animals used may have contributed to the diversity of the results.

This study was performed in order to evaluate the influence of the methods used upon the number of nuclei estimated in the liver after partial hepatectomy. Two different methods were used for the estimates and the experimentally found results regarding the number of nuclei and the amount of DNA per nucleus were compared with theoretically calculated values.

### Material and Methods

**Experimental Animals.** Five to seven weeks old male and female mice from an inbred C3H strain kept at the Institute were used, the males weighing 17–20 g and the females 15–17 g. The animals were kept under standard conditions of light and temperature and were given a standard laboratory diet ad libitum before and after the operation.

**Operation.** Partial hepatectomy was performed as previously described (Heby and Lewan, 1971). In sham operated animals the skin and abdominal wall were opened and the liver lobes handled. No significant differences regarding the number of nuclei per gram of liver were found between normal and sham-operated mice 24 hours after the operation. Normal mice were therefore used as controls.

Four hours before sacrifice 26 female hepatectomized mice were given colchicine (Merck), 1.5 mg/kg body weight.

**Autopsy.** The animals were killed by decapitation, at intervals up to 140 hours post hepatectomy, as shown in each figure. Registration of liver weight was performed as previously described and the results were in agreement with those already reported (Heby and Lewan, 1971).

**Mitotic Counts.** Sections for registration of mitotic figures were prepared as earlier described (Heby and Lewan, 1971). The number of mitoses per 100 nuclei in the colchicine treated mice,  $M_{CH}$ , was determined.

**Estimation of the Number of Nuclei and of the Mean Nuclear Diameter.** The number of nuclei was estimated in two ways, by counting in a microscopic field in sections from liver and by counting in a haematocytometer in homogenates from liver.

For estimation of the number of nuclei in a microscopic field, small pieces of liver from female mice were prefixed in 2.5% glutaraldehyde (2 h + 4°C) and postfixed in 1% osmium tetroxide solution, according to Millonig (1962) (1 h + 4°C). They were embedded in Vestopal, cut on an LKB ultratome in 1  $\mu$  sections, mounted on glass slides and stained with azur II (0.5%) and Methylene Blue (0.5%) in borate buffer (1%) according to the procedure of R  deberg (1967). The number of nuclei was estimated in 50 fields (1300 $\times$  magnification) for each animal. Two animals were examined at each point of investigation. The results are expressed as percentages of the mean number of parenchymal nuclei per field in sections from three normal livers, which was 15.4. The nuclear diameter was estimated in 100 nuclei per animal using an ocular micrometer.

For nuclear counts in homogenates, weighed pieces of liver were homogenized in 10 volumes of an acetate buffer (0.05 M NaAC, 0.07 M NaCl, 0.005 M CaCl<sub>2</sub>, 0.3 M sucrose, pH 4.0) with 10 strokes in a motor-driven glass-teflon homogenizer. Several homogenizing media were tested, including the TKM buffer (0.25 M sucrose, 0.025 M KCl, 0.005 M MgCl<sub>2</sub> in 0.05 M Tris-HCl, pH 7.5) used by Blobel and Potter (1966) and the citric acid buffer recommended by Price and Laird (1950). No significant differences were registered in the number of nuclei per gram of regenerating liver (72 hours), but maximal stability of the cell membrane seemed



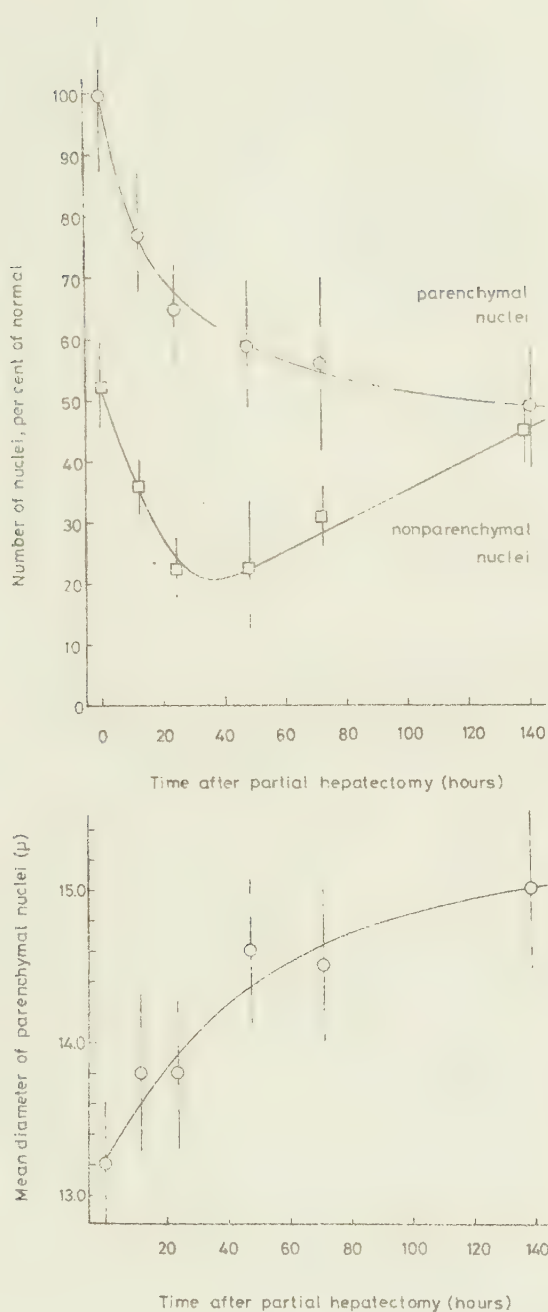


Fig. 1. Estimation in homogenate A. Changes in the number of parenchymal and nonparenchymal nuclei per gram of liver after partial hepatectomy expressed in per cent of the number of parenchymal nuclei in normal liver. Each point represents 6 animals. Mean  $\pm$  S. D. B. Changes in the mean diameter of parenchymal nuclei after partial hepatectomy. Each point represents 6 animals. Mean  $\pm$  S. D.





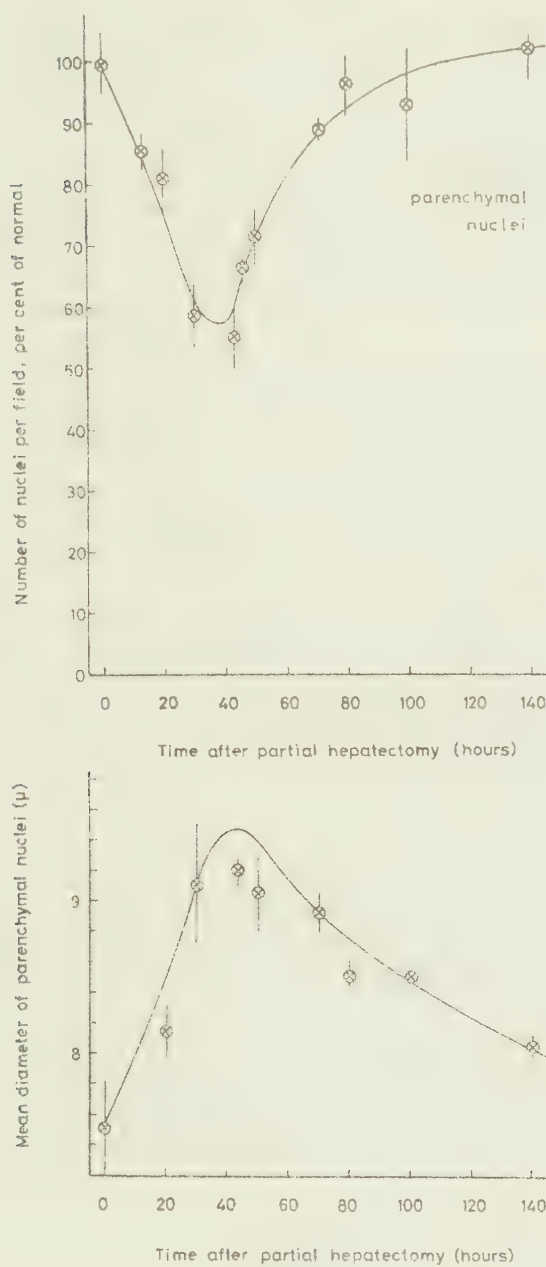


Fig. 2. Estimation in sections A. Changes in the number of parenchymal nuclei per unit volume after partial hepatectomy expressed in per cent of the number in normal liver. Each point represents the mean of two animals. The vertical bar represents the range. B Changes in the mean diameter of parenchymal nuclei after partial hepatectomy. Each point represents the mean of two animals. The vertical bar represents the range



to be obtained with the acetate buffer, pH 4. It was therefore chosen in order as far as possible to avoid nuclear fragmentation. The homogenate was diluted and stained with three parts of Türk's reagent and the number of nuclei estimated in a haematocytometer.

The liver homogenate was also examined in an inverse microscope (400 times magnification). A drop was placed on a coverglass and after sedimentation, nuclear diameters were estimated with the aid of an ocular micrometer. Irregularly shaped nuclei and small round nuclei with a diameter of less than  $8\mu$  were classified as nonparenchymal nuclei. The ratio between parenchymal and nonparenchymal nuclei, together with the value for the total number of nuclei per gram of liver, gave the absolute numbers of the different nuclei per gram. The numbers of both parenchymal and nonparenchymal nuclei per gram of liver during regeneration are expressed as percentages of the number of parenchymal nuclei per gram in normal liver. The mean diameter of the parenchymal nuclei was estimated at each point of investigation after the operation.

*Estimation of the Reproducibility of Homogenization.* When counting the nuclei in the haematocytometer, the percentage of nuclei free from cells was also registered.

*Calculation of the Amount of DNA and the Number of Nuclei per Liver.* The total amount of DNA in the liver was calculated from the amount of DNA per gram of liver and the total weight of the liver at each point of investigation (Heby and Lewan, 1971; Lewan, to be published). The total number of parenchymal nuclei in the liver was calculated from the number of nuclei per unit volume, as determined in sections (Fig. 2A), and the total weight of the liver at each point of investigation. The results are in agreement with those elsewhere reported, after allowing for possible volume changes during preparation (Brues, Drury, and Brues, 1936).

*Theoretical Calculations.* A theoretical curve for the number of parenchymal nuclei per liver during the regenerative period was drawn after graphical integration of the curve for mitotic index (Heby and Lewan, 1971). The integration was performed on the assumption that the mitotic duration was one hour (Fabrikant, 1967). A theoretical curve for the amount of DNA per liver during the regeneration was constructed by setting back the curve for the number of nuclei by 8 hours, on the assumption that DNA synthesis precedes mitosis by 8 hours (Fabrikant, 1967) and that the cells pass the S phase and the mitotic phase at the same speed.

*Calculation of the Amount of DNA per Parenchymal Nucleus.* The experimentally determined amount of DNA per parenchymal nucleus was calculated from the amount of DNA per gram of liver at each point of investigation (Heby and Lewan, 1971) and the number of nuclei per unit volume, as estimated in sections (Fig. 2A).

The theoretically calculated amount of DNA per parenchymal nucleus was arrived at by dividing the calculated amount of DNA by the calculated number of nuclei (Fig. 4A). The amount of DNA per nucleus during the experimental period is expressed as a percentage of the value in normal liver.

*Calculation of the Mitotic Duration.* The mitotic duration was calculated from the mitotic index in the regenerating liver and the number of mitotic figures collected after colchicine treatment, Table 1, on the assumption that the given dose of colchicine exerts a full block at metaphase for 4 hours (Tannock, 1967).

The mitotic duration was also calculated from the mitotic index in the regenerating liver and the number of nuclei as estimated in sections, Fig. 4A (Landing, 1949).

## Results

### *Number of Nuclei per Gram or Unit Volume of Liver and Mean Nuclear Diameter*

During the first 24 hours of mouse liver regeneration, there is a decrease in the number of nuclei per unit weight and unit volume of liver and an increase in mean nuclear diameter, Figs. 1A and B, 2A and B. If the estimation is performed on sections, a return of the number of nuclei and mean nuclear diameter towards the values in normal liver can be registered, Fig. 2A and B. The minimum in the



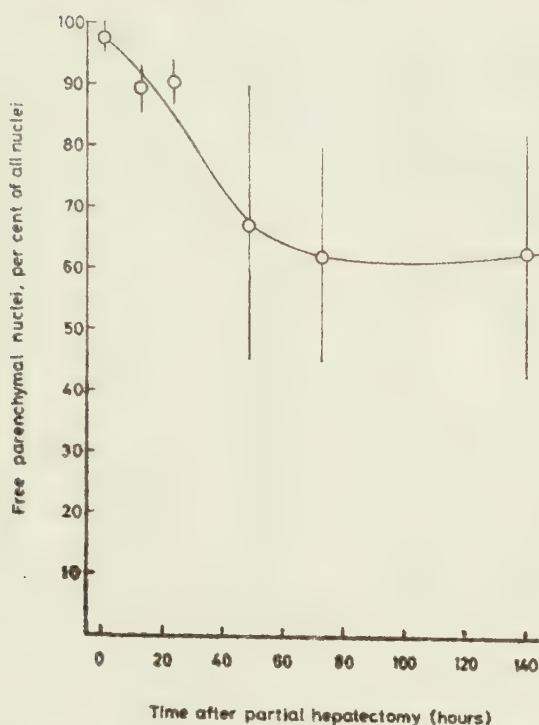


Fig. 3. Estimation in homogenate. Changes in the number of parenchymal nuclei free from cells after partial hepatectomy, expressed in per cent of the total number of nuclei. Each point represents the mean of 6 animals. Mean  $\pm$  S. D.

number of nuclei per unit volume is coincident with the maximum in mean nuclear diameter and occurs at the time of maximal mitotic index (Heby and Lewan, 1971). No return to normal values, either of the number of parenchymal nuclei per gram or of the mean diameter of parenchymal nuclei, can be registered during the experimental period if the estimates are made in liver homogenates, Fig. 1 A and B.

There is a decrease in the number of nonparenchymal nuclei per gram of liver during the first 48 hours of liver regeneration, Fig. 1 A. It then increases and almost reaches the normal value at the end of the period investigated.

#### *Reproducibility of Homogenization*

During regeneration, the state of the liver changes, resulting in poorer reproducibility of homogenization, Fig. 3. In homogenate from normal liver, the parenchymal nuclei appear free from cells. Very few whole cells with enclosed nuclei are seen. In homogenates from regenerating liver, however, few parenchymal nuclei free from cells are seen. There are many whole parenchymal cells with enclosed nuclei. Whole nonparenchymal cells are not found in homogenates either from normal or from regenerating liver.





Table 1. *Mitotic duration after partial hepatectomy*

| Time<br>after<br>operation<br><br>hours | Mitotic<br>index<br>cholch 4 h<br>$M_{CH}$<br>% | Mitotic<br>index <sup>a</sup><br>$M$<br>% | Mitotic<br>duration <sup>b</sup><br>according<br>to Tannock<br>hours | Mitotic duration <sup>c</sup><br>according<br>to Landing |       |
|-----------------------------------------|-------------------------------------------------|-------------------------------------------|----------------------------------------------------------------------|----------------------------------------------------------|-------|
|                                         |                                                 |                                           |                                                                      | time<br>interval                                         | hours |
| 20                                      | 0.1                                             | 0.2                                       | —                                                                    |                                                          |       |
| 30                                      | 0.7                                             | 0.8                                       | —                                                                    |                                                          |       |
| 40                                      | 1.0                                             | 2.7                                       | —                                                                    | 30-43                                                    | 4.4   |
| 43                                      | 1.6                                             | 3.7                                       | —                                                                    |                                                          |       |
| 46                                      | 16.1                                            | 3.6                                       | 1.1                                                                  | 43-46                                                    | 1.2   |
| 48                                      | 10.0                                            | (2.5)                                     | 1.3                                                                  | 46-50                                                    | 2.4   |
| 50                                      | 2.6                                             | 2.1                                       | 16.8                                                                 |                                                          |       |
| 55                                      | 9.3                                             | (1.5)                                     | 0.8                                                                  | 50-70                                                    | 1.6   |
| 66                                      | 5.9                                             | 1.4)                                      | 1.2                                                                  |                                                          |       |
| 70                                      | 2.8                                             | 1.5                                       | 4.6                                                                  | 70-80                                                    | 1.9   |
| 80                                      | 2.2                                             | 0.6                                       | 1.5                                                                  | 80-100                                                   | 1.4   |
| 100                                     | 1.1                                             | 0.6                                       | 4.8                                                                  |                                                          |       |
| 120                                     | 1.5                                             | (0.6)                                     | 2.6                                                                  | 20-140                                                   | 2.3   |

<sup>a</sup> Mitotic indices are from Heby-Lewan (1971). Values in parenthesis are approximated.

<sup>b</sup> Mitotic duration =  $\frac{M}{M_{CH} - M}$  (Tannock, 1967).

<sup>c</sup> Mitotic duration =  $\frac{ct^2 + 2at}{2 \ln \frac{N_A}{N_B}}$ , where  $c$  and  $a$  are constants derived from the linear

relationship between mitotic index ( $M$ ) and time ( $t$ ) that is supposed to be valid over a time interval, if short enough.  $N_A$  and  $N_B$  are the number of nuclei in the regenerating liver at the beginning and at the end of each interval, estimated in sections, Fig. 2A (Landing, 1948).

#### *The Number of Nuclei and the Amount of DNA, Calculated per Liver*

The number of parenchymal nuclei calculated per liver has a tendency to decrease in the regenerating tissue during the first 40 hours following hepatectomy, Fig. 4A. The amount of DNA per liver has already started to increase around 20 hours postoperatively. After a lag period of about 20 hours, the proliferation of nuclei starts. The rate of nuclear proliferation is then greater than the rate of DNA accumulation. Nuclear proliferation occurs at a slower rate than indicated by the mitotic index, assuming a mitotic duration of 1 hour. DNA synthesis does not seem to precede nuclear proliferation by a constant time interval of 8 hours, Fig. 4A.

#### *The Amount of DNA per Parenchymal Nucleus*

There is a transient increase in the amount of DNA per nucleus, Fig. 4B. The increase is larger, and starts earlier, than calculated on the assumption that the nuclei pass through the S and G<sub>2</sub> phases of the cell cycle at a constant speed, with eight hours between maximal DNA synthesis and maximal mitosis.



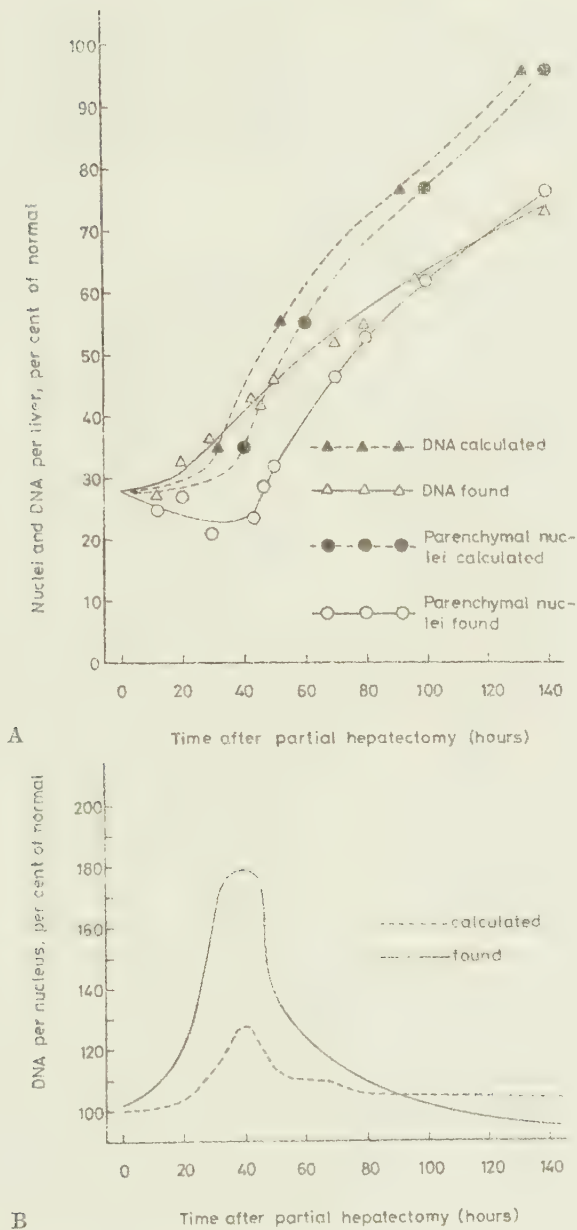


Fig. 4. A. Calculated and experimentally found changes in the total amounts of DNA and parenchymal nuclei per regenerating liver expressed in per cent of the values for normal liver. Each point represents the mean of two or three animals. B. Calculated and experimentally found changes in the mean amount of DNA per parenchymal nucleus after partial hepatectomy expressed in per cent of the amount per parenchymal nucleus in normal liver



### *Mitotic Duration*

Prior to the time of maximal mitotic index, colchicine has little stathmokinetic effect, Table 1. Little proliferation occurs at this time, Fig. 4A, in spite of the increasing number of mitotic figures, Table 1. These results indicate a long mitotic duration. After the time of maximal mitotic index, the results indicate a mitotic duration of between one and two hours, Table 1.

### *Discussion*

An increase in the degree of polyploidy during liver regeneration is widely accepted (Bucher, 1963, 1967; Grundman and Seidel, 1969) on the evidence of nuclear volume determinations and microspectrophotometric measurements in sections of liver (Grundman and Bach, 1960) and in liver cell suspensions (Nadal and Zaidela, 1966; Schopman and Delfgaauw, 1966). The sedimentation behaviour of liver cell nuclei (Johnston *et al.*, 1968) and biochemical determinations of DNA, combined with nuclear counts in liver homogenates, also indicate an increased polyploidy in the regenerating liver (Van Lancker and Sempoux, 1958; Tsuboi *et al.*, 1954). There are well-known methods for homogenizing normal liver (Dounce, 1955). Price and Laird (1950), on applying these to regenerating liver, considered little breakage of nuclei to occur during homogenization of the tissue. Contrary to other investigators (Brues, Drury, and Brues, 1936; Abercrombie and Harkness, 1951; Yokoyama *et al.*, 1953; Tsuboi *et al.*, 1954), these authors reported an increase in the number of nuclei per gram of liver during the period of most rapid proliferation, amounting to 140% of the number in normal liver.

The pronounced difference found in this study between the number of parenchymal nuclei as estimated in sections and homogenates, respectively, does, however, indicate considerable breakage of parenchymal nuclei at homogenization during and after the period of most rapid proliferation. When estimating the number of nuclei in sections, it cannot be excluded that differential shrinkage and swelling during the treatment of the specimens interfere with the results. However, the rapid proliferation of parenchymal nuclei observed during mouse liver regeneration is in accordance with findings on rat liver reported by Brues, Drury, and Brues (1936). These authors also performed their estimations in sections and took careful account of volume changes. Volume changes of blood vessels and biliary ducts may also interfere with estimations of the number of nuclei in sections. The volume of blood vessels and biliary ducts has, however, not been reported to change during liver regeneration (Aronsson, 1970). The spatial pattern of proliferation within the liver lobuli (Rabes and Tuczec, 1970) may have influenced the number of nuclei estimated in sections, but since the sections and the fields of examination within them were chosen at random, this source of error should be small.

A selective breakage of small, newly formed nuclei seems probable, since differences depending on the method used for nuclear counts are not pronounced until after mitosis. This assumption is supported by the fact that the mean diameter of the nuclei in homogenates increases rather than decreases after mitosis. The fragmentation of the nuclei during regeneration is contradictory to the increased stability of the cells. There may, however, be a more pronounced





connection between nuclear membrane and endoplasmatic reticulum in newly formed growing cells, rendering it difficult to disrupt the cell membrane without concomitant destruction of the nucleus. The great difference between normal and regenerating liver, with regard to the stability of cells and nuclei, renders comparisons performed in homogenates unreliable.

The early and large increase in the amount of DNA per nucleus is dependent both upon the decrease in the number of parenchymal nuclei prior to mitosis, and upon the increase in the amount of DNA during this period. The same tendency regarding the change in amount of DNA per nucleus in regenerating mouse liver has been found by other investigators (Yokoyama *et al.*, 1953; Tsuboi *et al.*, 1954). In the regenerating rat liver, the increase does not start until 12 hours postoperatively but the amount of DNA reaches 160–180% of the normal value just before mitosis (Van Lancker and Sempoux, 1958; Orlova and Rodionov, 1970). It cannot be excluded that an excessive decrease in the number of non-parenchymal nuclei (Daoust, 1958; Edwards and Koch, 1964), and transport of chromatin products from degraded lymphatic tissues (Bryant, 1963) influence estimations of the amount of DNA per parenchymal nucleus during this period.

The mice were in poor condition prior to the time of mitotic activity, but recovered at the time when mitosis started. This is indicated by changes in body weight (Jaffe, 1954; Lewan to be published) and fat content of the parenchymal cells (Tsuboi, 1954; Tygstrup, 1967). The rapid decrease in thymus weight after partial hepatectomy (Lewan to be published), which is common in stress, and the alteration of the cellular membrane indicate homeostatic changes (Ballard and Tomkins, 1969), as have been suggested by several authors (Grundman and Seidel, 1969; Rabes *et al.*, 1970; Rabes and Tuzek, 1970; Echave Llanos, 1970).

The metabolic state of the liver may determine the degree of binuclearity of the parenchymal cells (Wilson and Leduc, 1948; Bucher and Suppan, 1967) and thus explain the decrease in amount of parenchymal nuclei during early regeneration. Impaired carbohydrate metabolism in combination with modified homeostasis may influence the mitotic rate and the duration of the different phases of the cell cycle (Bullough, 1962; Bullough and Laurence, 1968; Brown and Berry, 1968) during the regenerative process.

The mitotic duration, as determined in this study, is longer than reported by others, as reviewed by Fabrikant (1968). However, it must be stressed that no analysis (Tannock, 1967) of the stathmokinetic effect of colchicine in the regenerating liver has been performed and that deviations in the mitotic index affect the values for mitotic duration with any of the methods used. An examination of the mitotic index at closer intervals may, because of the circadian rythm (Echave Llanos and Bordin, 1963; Echave Llanos *et al.*, 1967, 1971), have changed the proportions between the calculated and the experimentally found numbers of nuclei in this investigation.

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The amount of DNA per nucleus during mouse liver regeneration<sup>x</sup>

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Page heading: DNA per nucleus in regenerating liver.





## Summary

The number of nuclei and the amount of DNA were determined in homogenates from normal and regenerating liver of mice at intervals upto 120 hours after hepatectomy. The mean amount of DNA per liver nucleus increased between 25 and 50 hours after hepatectomy to 200 per cent of the value in normal mouse liver and remained constant for the remainder of the analysis period. As found after administration of vinblastine, a large number of nuclei were undergoing mitosis at the time when the amount of DNA per nucleus increased.

The number of nuclei and the amount of DNA were also determined in the pellets resulting from ultracentrifugation of the analysed homogenates. A good correlation was found between the percentage of sedimented nuclei and the percentage of sedimented DNA, indicating that the nuclei existed in an unbroken state in the homogenates. At analysis of the diameter of the parenchymal nuclei a decrease in the number of small nuclei (9-14  $\mu$ ) was found while nuclei having a diameter greater than 19  $\mu$  appeared.

The number of nuclei per volume of liver was not found to change during regeneration, when analyses were made on paraffin embedded sections. Possible reasons for the differences in the number of liver nuclei measured per volume during regeneration are discussed.

## Introduction

During liver regeneration following partial hepatectomy there is a hypertrophy of the parenchymal cells followed by DNA synthesis and mitotic activity (Bucher, 1963, 1967; Grundman and Seidel, 1969; Bengmark, 1970). The mitoses may lead to true proliferation of nuclei (Brues et al., 1936) or to nuclei with a higher degree of polyploidy (Nadal and Zaidela, 1966). The increase in polyploidy is difficult to calculate, as the amount of DNA per gram of liver tissue is fairly constant during liver regeneration (Lancker and Sempoux, 1958; Tsuboi



et al., 1954), but reports regarding the variation in the number of nuclei do not entirely agree (Brues et al., 1936; Lancker and Sempoux, 1958; Price and Laird, 1950; Landing et al., 1949; Abercrombie and Harkness, 1951; Harkness, 1952; Tsuboi et al., 1954). The reason for this may partly depend on whether the number of nuclei is estimated in homogenates or whether it is estimated on sections (Lewan, 1971).

The nuclei in homogenates from normal and regenerating liver have therefore been studied further and a comparison has been made between the number of nuclei found in vestopal embedded sections and that found in paraffin embedded sections. Furthermore, the mitotic indices were determined. Mitotic indices were also determined 4 hours after the administration of vinblastine, a metaphase arresting agent.

#### Material and methods

Experimental animals. Young male and female C3H mice from an inbred strain kept at the institute were used for the experiments. The female mice weighed 15-17 g, the male 18-21 g. They were kept at standard conditions regarding light and temperature and were fed on a standard laboratory diet.

Partial hepatectomy. Partial hepatectomy was performed essentially according to Higgins and Andersson (1931) as described earlier (Heby and Lewan, 1971).

Autopsy and tissue treatment. The animals were killed by decapitation at intervals upto 140 hours post hepatectomy. One series of mice was given vinblastine, 3 mg/kg body weight, 4 hours before autopsy. The livers were dissected out and those used for biochemical analyses were frozen at  $-20^{\circ}\text{C}$  until preparation. From mice to be used for histological examinations samples of liver for fixation were taken at autopsy.

Homogenization and isolation of nuclei. The livers were homogenized and the nuclei isolated essentially according to Blobel and Potter (1966). One gram of liver tissue was homogenized in 3 ml of TKM-buffer (0.25M



sucrose, 0.05M Tris, 0.025M KCl, 0.005M  $MgCl_2$ ) with ten strokes of the pestle in a glass-teflon homogenizer. The homogenate was underlayered with 0.3M sucrose in TKM-buffer, and the nuclei isolated by centrifugation for one hour at  $8 \times 10^5 g_{av}$  in an MSE superspeed 50 centrifuge. The isolated nuclei were resuspended by homogenization in TKM-buffer.

Determination of the number of nuclei and the amount of DNA in the homogenates. Samples were taken from the liver homogenate before ultracentrifugation and from the suspension of the isolated nuclei after ultracentrifugation. The nuclei were stained in aceto-orcein (Kurnick-Ris, 1948) and counted in a haematocytometer.

The amount of DNA was analysed with diphenylamine as described earlier (Heby and Lewan, 1971).

The amount of DNA per nucleus in the liver homogenate and the amount of DNA in the suspension of the isolated nuclei were calculated.

The number of nuclei and the amount of DNA in the suspension of the isolated nuclei were expressed as percentages of the number of nuclei and the amount of DNA in the homogenate respectively.

Nuclear diameter. The diameter of 60-80 parenchymal nuclei in homogenates from normal and regenerating liver were measured in samples stained with Türk's reagent as described previously (Lewan, 1971). The diameter of 50 nuclei in sections from paraffin embedded livers of 5 normal mice and of 4 mice 140 hours after hepatectomy was also measured.

Number of nuclei in sections and mitotic index. Pieces of liver were fixed in 4%  $CaCO_3$  buffered formalin, embedded in paraffin, cut in 5  $\mu$  sections and stained with haematoxylin-eosin. The number of parenchymal nuclei per microscopic field was counted in 50 fields at 1250 x magnification, by two different people.

For determination of the mitotic index, 1000 parenchymal nuclei were examined. Metaphases and anaphases were counted as mitoses. The mitotic index was given as the number of mitoses per 100 nuclei. In vinblastine treated animals the mitoses were arrested and identified as areas of dark coloured dots.





## Results

DNA per nucleus. The average amount of DNA per nucleus in the liver homogenate, see Fig. 1A, started to increase already 24 hours after hepatectomy. It doubled at 48 hours and did not significantly decrease during later stages of regeneration.

The amount of DNA per nucleus as found in a suspension of isolated nuclei, see Fig. 1B, pursues the same general course as that in the liver homogenate. The variations are however larger.

Yield at ultracentrifugation. There is a very good agreement between the percentage amount of DNA and the percentage number of nuclei recovered in the suspension of isolated nuclei, Fig. 2. The percentage yield of nuclei and of DNA decrease during the regenerative period. During the analysis period an increasing number of very large nuclei and whole cells could also be identified in the supernatant after the ultracentrifugation.

Nuclear diameter. There is a large decrease in the number of small nuclei having a diameter between 9 and 14  $\mu$  during the period of investigation, Fig. 4. The number of nuclei having a diameter of 14 to 19  $\mu$  is almost unchanged. Nuclei having a diameter larger than 19  $\mu$  appear and increase in number during the regeneration. The mean diameter of parenchymal nuclei in sections from paraffin embedded normal livers was 6.8 (6.5; 7.1; 6.8; 7.0; S.E. less than 0.3). At 140 hours after hepatectomy the diameter was 8.2 (8.4; 8.2; 8.1; 8.1; S.E. less than 0.4).

Number of nuclei per field in sections. The number of nuclei per microscopic field in paraffin embedded sections did not alter significantly, Fig. 3. These results were obtained by two different persons working independently of each other.





Mitotic index. Mitotic activity was found in animals 48 hours after the operation and at later stages, Table 1. The individual variations were large. The number of nuclei entering mitoses per hour was large at 48 to 72 hours after the operation as judged from the experiments with vinblastine treated mice. A possible daily rhythm was indicated by the somewhat lower value in the evening 60 hours after the operation.

### Discussion

The lasting increase in polyploidy during mouse liver regeneration found in this study is in agreement with analyses on rat liver when microspectrophotometric and karyometric methods (Grundmann and Bach, 1960; James et al., 1966) were used and is also in agreement with analyses on regenerating mouse liver when biochemical methods were used (Yokoyama et al., 1953; Tsuboi et al., 1954).

The DNA-synthesis, which is known to start around 30 hours after partial hepatectomy in the mouse (Jardetzky et al., 1956; Bade et al., 1966) obviously leads to an increase in the amount of DNA per nucleus to approximately 200% of the value in normal liver, Fig. 1A. The number of nuclei entering mitoses per hour starts to increase between 36 and 48 hours after the operation (Table 1). As the mitotic activity does not bring about a decrease in the amount of DNA per nucleus, it is reasonable to conclude that the mitoses between 30 and 50 hours after the operation result in a higher ploidy of the nuclei rather than in nuclear proliferation (Beams and King, 1942; Nadal and Zaidela, 1966). However, DNA-synthesis (Jardetzky et al., 1956) and mitotic activity (Table 1; Wilson et al., 1953) continue as the liver grows without further increase in polyploidy. This may indicate that the DNA-synthesis and mitotic activity between 50 and 140 hours after hepatectomy lead to proliferation of nuclei, and this has also been found



earlier (Yokoyama, 1953). Biochemical analysis, however, can only give mean values, and large variations may be found within the lobuli (Rabes and Tucek, 1970) and in the daily rhythm of DNA-synthesis and mitotic activity (Russo and Llanos, 1964). Another source of error is that the proportion between parenchymal and non-parenchymal nuclei is not quite constant (Wilson, 1954; Edwards and Koch, 1964).

As only small variations are found in the DNA concentration during liver regeneration (Tsuboi et al., 1954; Lancker and Sempoux, 1958; Lewan, 1972) a persistent high amount of DNA per nucleus must be correlated with a persistent low number of nuclei per gram or volume of liver tissue. This is easily seen in liver homogenates (Tsuboi et al., 1954; Yokoyama, 1953; Lewan, 1971), though not in sections embedded in paraffin, Fig. 3, or vestopal (Lewan, 1971). This may be due to a different mode of proliferation with age (Bucher and Glinos, 1950) or sex of the compared mice, as the analyses were not performed on the same animals.

The different batches of mice used for the investigations may have suffered to varying degrees from the hepatectomy. Mitotic activity is inhibited by fat feeding (Brues et al., 1936; Talarico et al., 1971) and DNA-synthesis is delayed by glucocorticoid hormones (Feigelson et al., 1962; Rizzo et al., 1971). RNA-synthesis on the other hand is less influenced by the nutritional state (Stenram et al., 1971) and is enhanced by glucocorticoids (Feigelson et al., 1962). The increase in the number of binucleate cells and polyploidy during normal liver growth appears preferentially around the central vein in the lobule (Sulkin, 1943) and are supposed to be dependent upon liver growth (Nadal and Zaidela, 1966).

Beside the physiological problem of synchronizing the regenerative process in different animals, several technical problems are also met with during work with regenerating liver. These arise due to fat infiltration, which in mouse liver is maximal between 24 and 48 hours after hepatectomy (Tsuboi et al., 1954) and also due to altered



proportions between the number of small and large nuclei as indicated in Fig. 4. The more variable nuclear size in the normal liver of the mouse as compared to that of the rat (Epstein, 1967; Nadal and Zaidela, 1966; Gerhard et al., 1971; Tongiani and Puccinelli, 1970), probably leads to greater sources of error in the mouse than in the rat at nuclear counts on sections, independent of embedding material.

The well known hypertrophy of the liver cells during the pre-mitotic period of the regenerative process in the rat (Brues et al., 1936; Harkness, 1952) is not easily seen on paraffin embedded sections from regenerating mouse liver, Fig. 3. Attention has not, however, been paid to the shrinkage (Brues et al., 1936; Landing et al., 1949) of the tissue during preparation. It may be pronounced in the lipophile paraffin as the liver is heavily infiltrated with fat prior to mitotic activity. In vestopal embedded sections, however, a pronounced hypertrophy of the cells in the regenerating liver of the mouse was found prior to proliferation (Lewan, 1971). It is possible, that the vestopal embedded sections are not able to stain as well when the tissue contains fat. In that case the number of nuclei will be underestimated and their volume overestimated in vestopal embedded sections during the fatty period of liver regeneration, 0-80 hours after the operation.

At 140 hours after hepatectomy the number of nuclei per microscopic field in regenerating liver does not seem to differ significantly from that found in normal liver, Fig. 3, (Abercrombie and Harkness, 1951; Lewan, 1971). A correction for the nuclei only partly lying within the section can be made by multiplying the number of nuclei with  $\frac{t}{t + d}$  where  $t$  is the thickness of the section and  $d$  is the mean diameter of the nuclei (Brues et al., 1936; Landing et al.,





1949). As  $t$  equals 5 and  $d$  increases from around 13 to 15 (Lewan, 1971) during the regeneration, no significant difference is noted.

The decreasing yield at ultracentrifugation of DNA and of nuclei, Fig. 2, depends on the presence of an increasing number of large nuclei and whole cells in the homogenates during regeneration. These may have difficulties in penetrating the discontinuous gradient at ultracentrifugation. An increased number of whole cells has been found in  $\text{Ca}^{2+}$  containing homogenates from regenerating liver as compared to normal liver (Lewan, 1971). This may also hold true in the presence of  $\text{Mg}^{2+}$ . The good correlation between the amount of DNA and nuclei sedimented at ultracentrifugation at each interval of analysis indicates that the nuclei have not been damaged at homogenization in the TKM-buffer used.

It is obviously difficult to obtain concurring results regarding the number of nuclei per gram or volume of liver tissue found in sections and that found in homogenates during mouse liver regeneration. Analysis of large sections and homogenates of liver samples from the same animal will probably produce better agreement than has hitherto been the case. Attention should be paid to shrinkage at embedding and to fields of non-parenchymal tissues within the sections, and moreover to changes in the nuclear volumes during regeneration.





Fig. 1A. Mg DNA per nucleus  $\times 10^{-8}$  measured in homogenates from regenerating liver.

Fig. 1B. Mg DNA per nucleus  $\times 10^{-8}$  measured in suspensions of nuclei isolated from the homogenates in Fig. A. Each point represents 2-9 experiments. Mean  $\pm$  S.D.

Fig. 2. The amount of DNA and the number of nuclei recovered in the pellet after ultracentrifugation as percentages of the DNA and the nuclei in the homogenates respectively. Each point represents 2-9 experiments. Mean  $\pm$  S.D.

Fig. 3. The number of parenchymal nuclei per microscopic field in sections from paraffin embedded pieces of normal and regenerating livers. Each point represents 50 fields on one section from each animal. Mean  $\pm$  S.D.

Fig. 4. The number of parenchymal nuclei of different sizes per gram of normal and regenerating liver. The total number of parenchymal nuclei per gram of normal liver was said to be 100. Each histogram represents the mean of 6 animals.

Table 1. Mitotic index in the liver after hepatectomy and also after hepatectomy with administration of vinblastine 4 hours before autopsy.



Fig. 1A

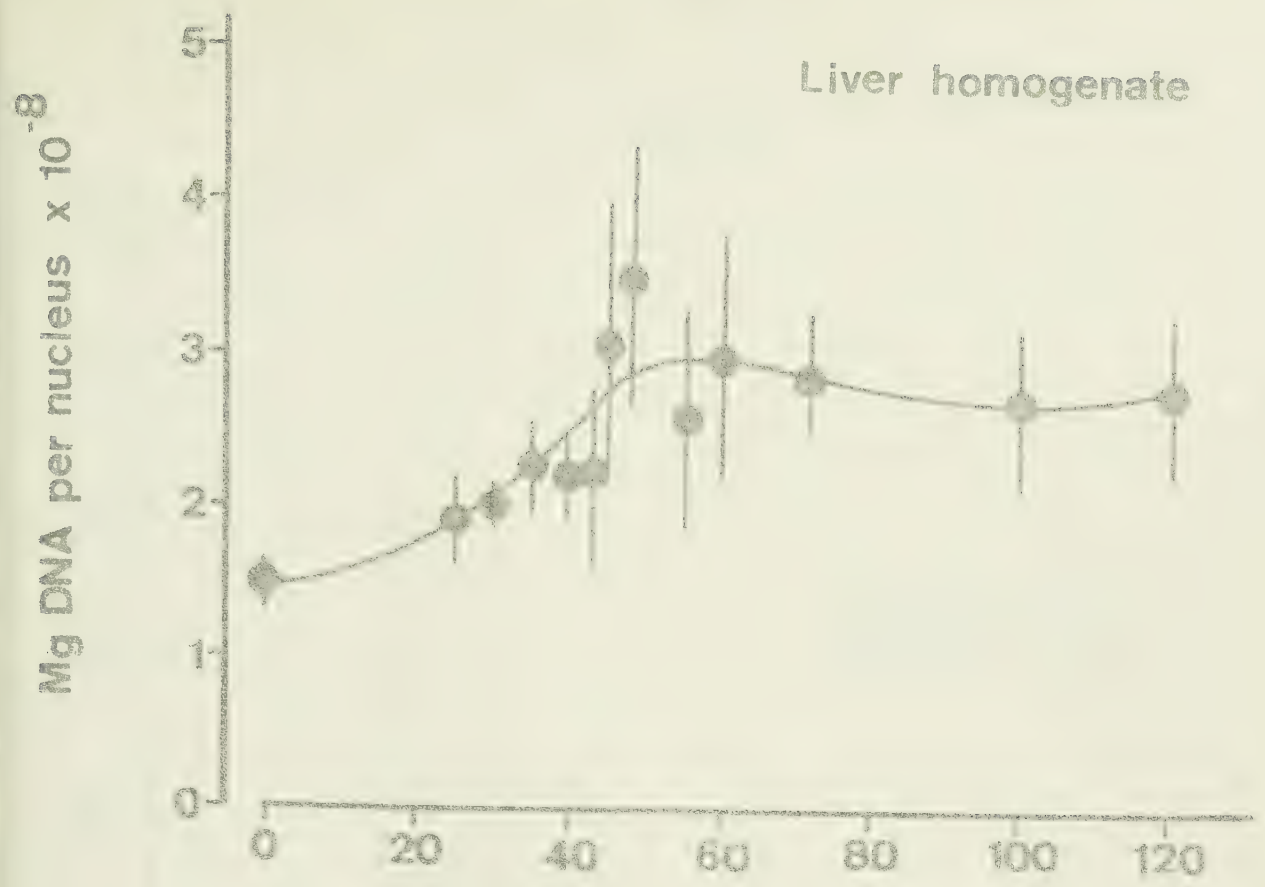


Fig. 1B

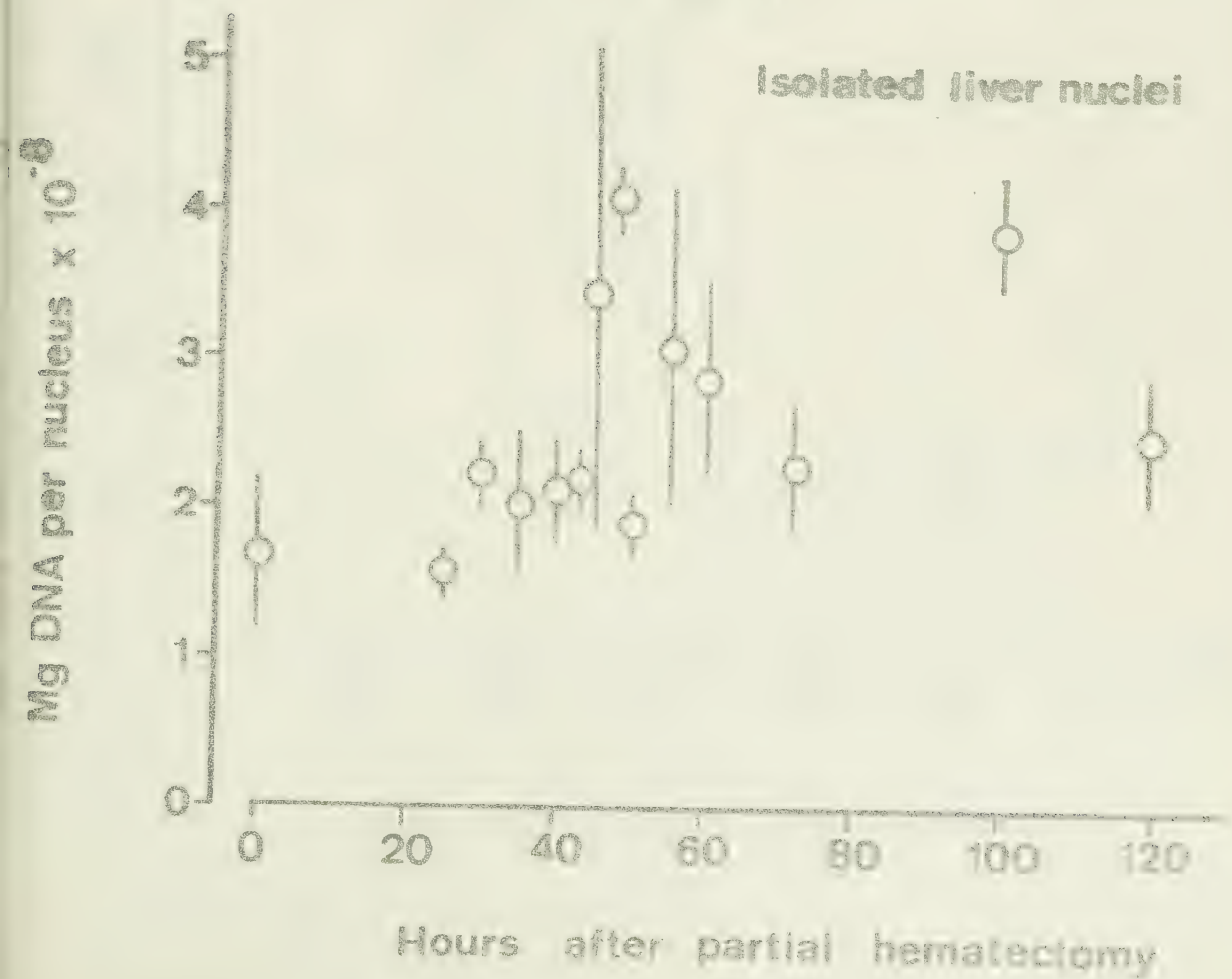
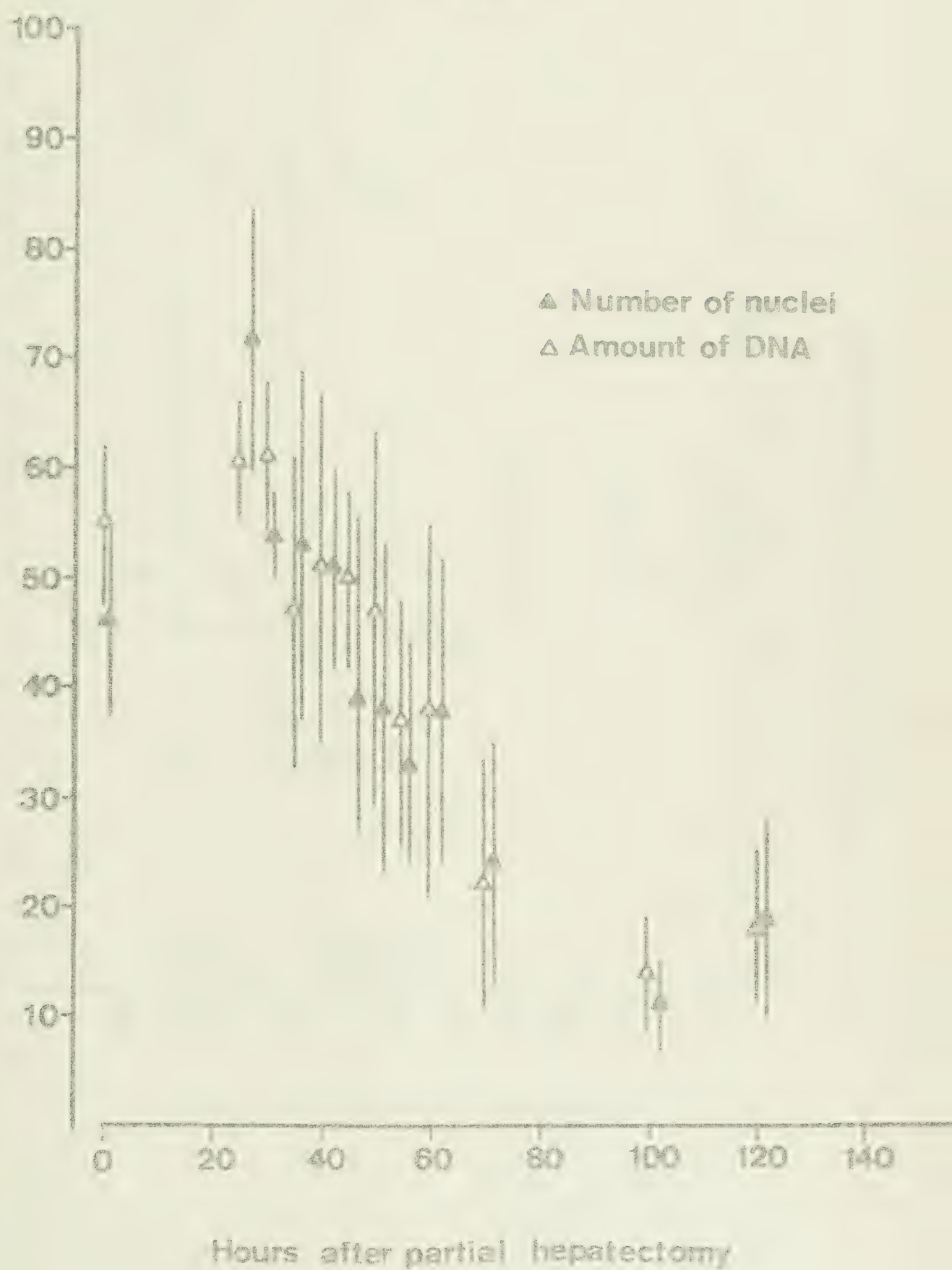
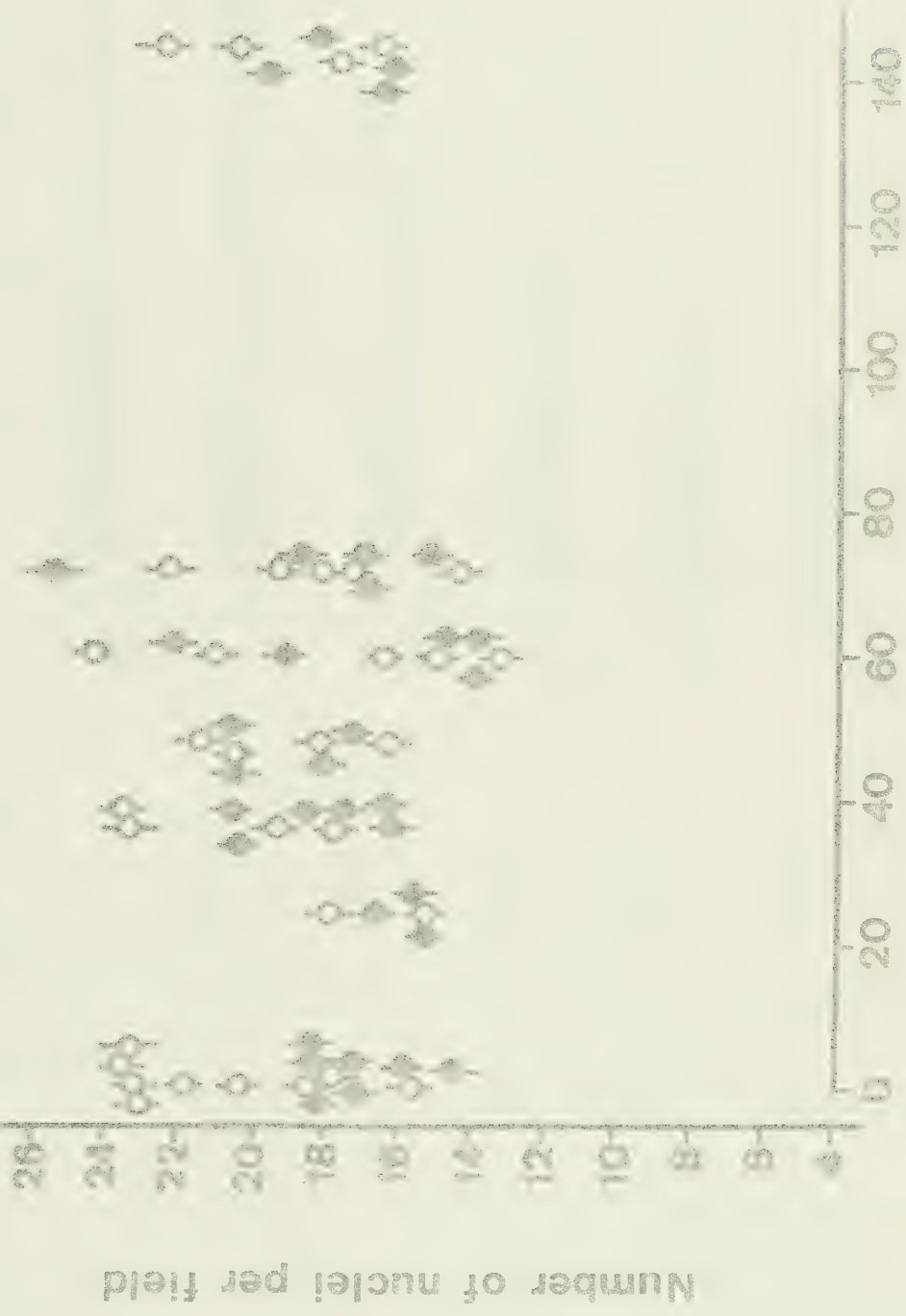




Fig. 2







Hours after partial hepatectomy





Fig. 4

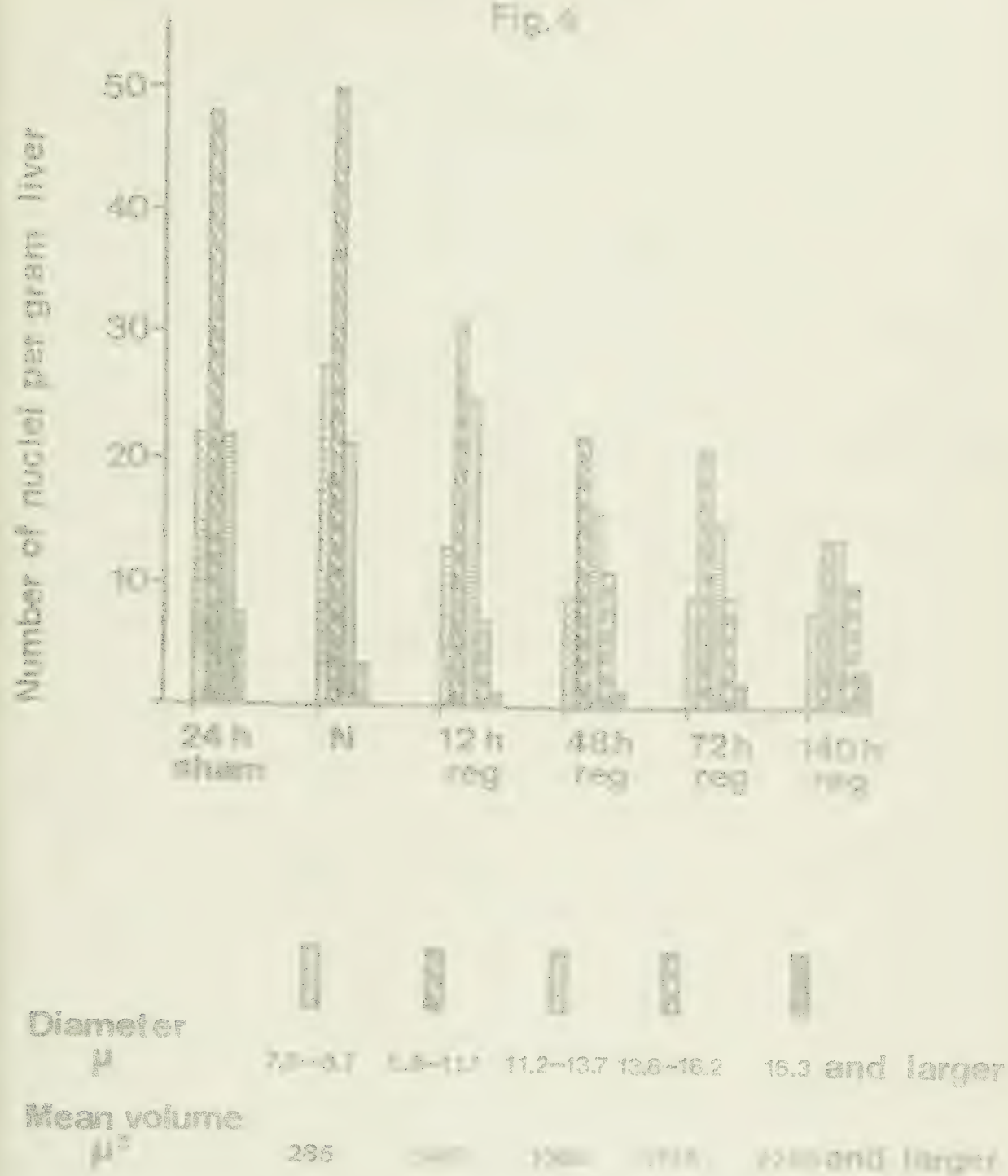




Table 1. Mitotic index

|      | 1) Normal<br>n mice | Time after partial hep |   |   |   |   |
|------|---------------------|------------------------|---|---|---|---|
|      |                     | 0                      | 1 | 2 | 3 | 4 |
| mice |                     |                        |   |   |   |   |
|      |                     |                        |   |   |   |   |

1) Number of animals



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The stathmokinetic effect of vinblastine during mouse liver regeneration<sup>x</sup>

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Page Heading: Influence of vinblastine during liver regeneration.





### Summary

Vinblastine (1.5-9 mg/kg body weight) was given to mice 70 hours after partial hepatectomy. At autopsy 4 hours later only arrested mitoses were found. An inverse relationship between the dose of vinblastine and the number of mitoses found after four hours was indicated.

The mitotic indices in the liver 24, 48, 72, and 144 hours after hepatectomy with and without treatment with vinblastine (3 mg/kg body weight) were compared. No stathmokinetic effect of vinblastine could be found at 48 hours but it was observed 72 hours after hepatectomy. This may depend entirely upon individual variations in the mitotic response to hepatectomy or may possibly be due to a longer mitotic duration at 48 hours after hepatectomy than at 72 hours. Alternatively the liver cells may be more sensitive to the poisonous action of vinblastine at 48 than at 72 hours after the operation.

### Introduction

The process of liver regeneration after partial hepatectomy is characterized by DNA-synthesis and increased mitotic activity (Bucher 1963, 1967; Grundmann and Seidel, 1969; Bengmark, 1970). The mitoses lead to proliferation of new nuclei or to an increased polyploidy of the nuclei (Beams and King, 1942). The number of nuclei entering mitosis per hour at different intervals after hepatectomy may possibly be estimated with the stathmokinetic method (Dustin, 1959). Together with microspectrophotometric (Grundman and Bach, 1960) and biochemical (Lancker and Sempoux, 1958) analyses of the degree of polyploidy among the liver cell nuclei, information about the mitotic rate should enable a calculation of the extent to which the mitotic activity after partial hepatectomy contributes to an increasing polyploidy.



Proper use of the stathmokinetic method presupposes that the influence of/ the drug used and the duration of its stathmokinetic effect in are/ the analysed tissue known (Tannock, 1967). In this preliminary study the effect of vinblastine on the mitotic index of regenerating mouse liver was evaluated.

#### Material and methods

Forty-seven young female Swiss albino mice, weighing 26-30 g, from Anticimex, Uppsala, were used for the experiments. Partial hepatectomy, essentially according to Higgins and Andersson (1931), and sham-operation were performed as described earlier (Heby-Lewan, 1971; Lewan 1971). The animals were kept under standard conditions of light and temperature and were fed on a standard laboratory diet ad libitum before and after the operation, which was performed between 8 and 10 a.m. Vinblastine (Lilly), 1 mg/ml or 0.5 mg/ml in 0.9% NaCl was sprayed intraperitoneally in 33 mice at 7 a.m. They were killed by decapitation four hours later.

The influence of different amounts (1.5 mg - 9 mg/kg body weight) of vinblastine on the mitotic index of the liver was tested in normal mice and in hepatectomized mice around 72 hours after operation. The influence of a constant amount of vinblastine (3 mg/kg body weight) was tested on the mitotic index of the liver of normal mice and of the liver of mice around 24, 48, 72, and 144 hours after partial hepatectomy.

Animals not treated with vinblastine were killed at 9 a.m.

A part near the tip of the posterior part of the right lateral liver lobe in all animals was fixed in  $\text{CaCO}_3$  buffered 4% formalin for at least 24 hours at room temperature. They were embedded in paraffin, cut in 5  $\mu$  sections and stained with haematoxylin and eosin. After observing 1000 parenchymal nuclei, the mitotic index was determined as the number of mitoses per 100 nuclei.

In animals not treated with vinblastine metaphases and anaphases were counted as mitoses.



## Results and Discussion

The risk involved in working with a stathmokinetic agent in too large a concentration even during the short period of four hours was indicated in this study. The effect of the drug also seemed to be very different at early stages of liver regeneration (48 h) than at later stages (72 h, 144 h).

In animals treated with vinblastine, all mitoses in liver parenchymal cells were abnormal, Fig. 1, indicating an effective metaphase arrest.

Vinblastine is considered to exert its antimitotic effect by acting on microtubular protein (Malawista et al., 1968). It may also be bound to other proteins (Wilson et al., 1970), inhibit RNA and DNA synthesis (Richards, 1968; Luyckx and Lancker, 1966) and have various common metabolic effects, which, depending on the dose, may be lethal after a few days (Lancker et al., 1966). The very high doses of vinblastine tested in this study were chosen to ensure a metaphase arrest in 4 hours (Elgio, pers. commun). Around 72 hours after hepatectomy doses of 9 and 6 mg vinblastine per kg body weight may partly have inhibited cells in the liver from entering mitoses even in the short interval of four hours. Around forty-eight hours after hepatectomy vinblastine had little stathmokinetic effect. This may have depended on an increased mitotic duration (Iversen and Eversen, 1962) or on a greater sensitivity of the liver cells to the poisonous action of vinblastine at 48 than at 72 hours postoperatively.

After administration of vinblastine somewhat larger concentrations of the poison have been found in the liver and intestine than in other organs in the body, and the excretion of vinblastine occurs through the bile ducts (Beer and Richards 1963-64). Thus, there may have been a greater sensitivity to the poison during early regeneration, when liver DNA-synthesis and proliferation has only just started, as compared with later stages when there are more functioning cells in the liver.



It must be emphasized, however, that the low mitotic index after vinblastine treatment around 48 hours after partial hepatectomy may also have been a function of less good synchrony (Markelova and Liozner, 1969) among the liver cells at the time of spraying in the analysed animals. Moreover a deviating daily rhythm (Russo and Llanos, 1964) or the intralobular localization of the analysed liver sections (Rabes and Tuczec, 1970) may have influenced the results.





Table I. Percentage of parenchymal nuclei in mitoses.

| Number of animals | State of mice     | Mg Vinblastine per kg body w. 4 h before killing | % Mitoses <sup>1)</sup> | Number of animals | Hours after operation | Mg Vinblastine per kg body w. 4 h before killing |
|-------------------|-------------------|--------------------------------------------------|-------------------------|-------------------|-----------------------|--------------------------------------------------|
| 2                 | normal            | 9                                                | 0                       | 2                 | 24 h shamop.          |                                                  |
| 2                 | 72 h after hepat. | 9                                                | 0.3;1.3                 | 1                 | 24 h shamop.          |                                                  |
| 2                 | normal            | 6                                                | 0                       | 3                 | 24 h hepat.           |                                                  |
| 2                 | 72 h after hepat. | 6                                                | 1.1;1.3                 | 4                 | 24 h hepat.           |                                                  |
| 4                 |                   |                                                  |                         | 3                 | 48 h hepat.           |                                                  |
| 4                 | 72 h after hepat. | 3                                                | 3.2;2.0;1.2; 2.1        | 4                 | 48 h hepat.           |                                                  |
| 2                 | normal            | 1.5                                              | 0                       | 4                 | 72 h hepat.           | 0.5 <sup>+</sup> 0.4                             |
| 2                 | 72 h after hepat. | 1.5                                              | 0.9; 3.0                | 3                 | 72 h hepat.           | 0.2 <sup>+</sup> 0.3                             |
|                   |                   |                                                  |                         | 3                 | 144 h hepat.          | 0                                                |
|                   |                   |                                                  |                         | 3                 | 144 h hepat.          | 0.4 <sup>+</sup> 0.2                             |

2) Mean + S.D.



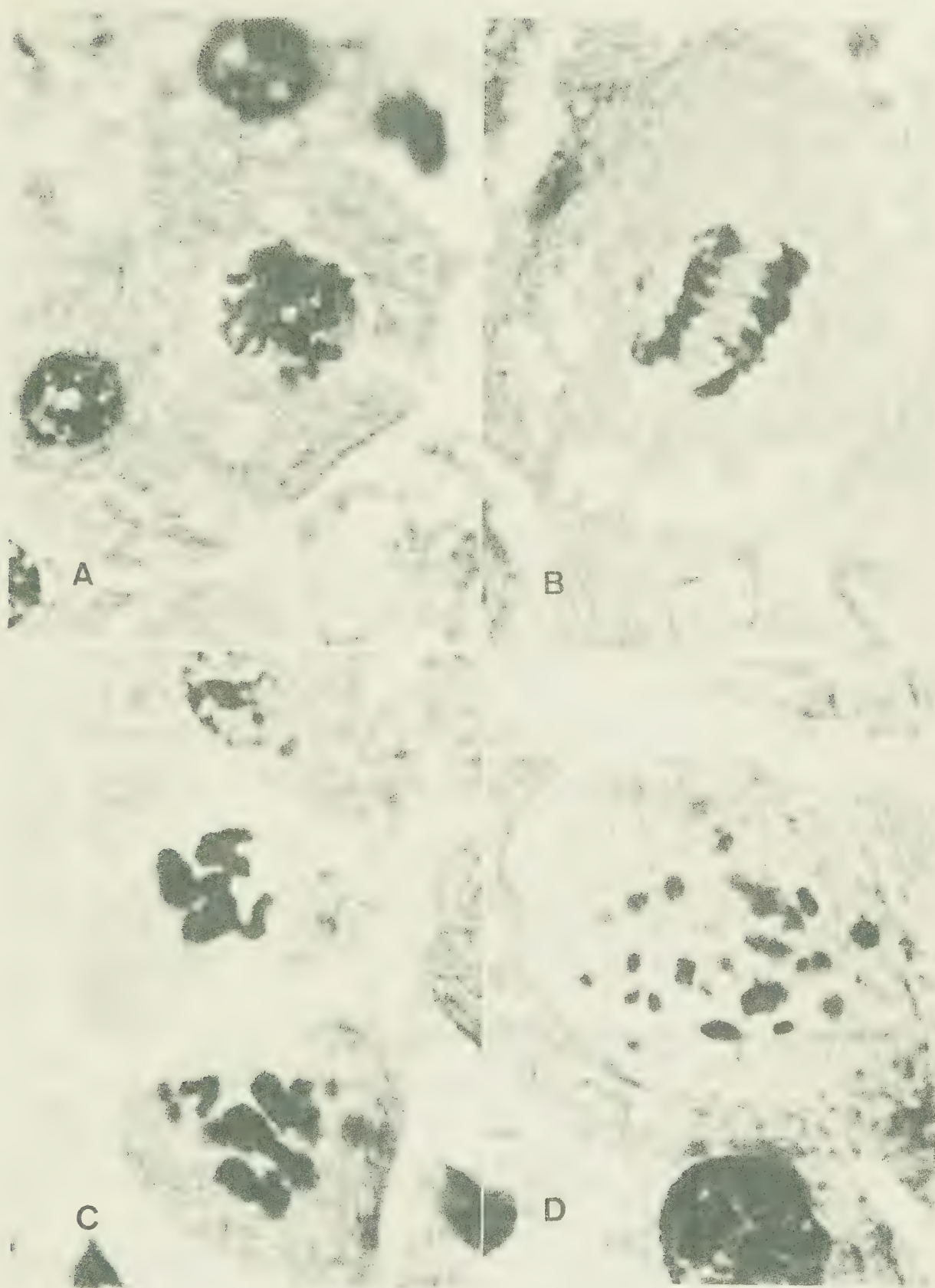


Fig. 1. A, B Liver mitoses 72 hours after hepatectomy  
 C, D Liver mitoses 72 hours after hepatectomy and 6 hours  
 after vin. blastine administration



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Protein content of liver cell nuclei isolated after partial hepatectomy<sup>x</sup>

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## Summary

An analysis was carried out of cell nuclei isolated from liver. Their content of RNA and of neutral soluble, acid soluble, alkali soluble and residual proteins, was measured relative to DNA.

A comparison was made between the values found in normal mice, and in mice 0-40 hours after partial hepatectomy or shamoperation.

Indications were found for increased amounts of RNA, neutral soluble and acid soluble protein in the nuclei of regenerating livers as compared to the liver nuclei from sham operated animals. These findings may be related to gene activation but may also be contributed to by an increased adherence of the cytoplasm to the nuclei during the lipid rich premitotic period of liver regeneration.

Electrophoresis was carried out on a chromatin acidic protein fraction obtained by gradient centrifugation of a CsCl extract from normal liver cell nuclei. Using polyacrylamide gels the electrophoretic properties of these proteins were similar to those of serum proteins.

## Introduction

The well regulated growth of the liver remaining after partial hepatectomy is characterized by an increased synthesis of RNA, protein and subsequently DNA (for review see Bucher, 1963, 1967; Grundmann and Seidel, 1969; Bengmark, 1970). The possible appearance of new types of RNA within an hour after operation indicate an early activation of repressed chromatin (Church and McCarthy, 1967; Mayfield and Bonner, 1972). About 20 hours after the operation the increased activity of enzymes involved in DNA-synthesis and mitotic activity may be mediated by newly synthesized RNA molecules and thus also indicate gene activation





(Lancker, 1970). As the histones and nuclear non-histone proteins are believed to play a role in gene regulation their quantity and quality have been analysed in several different systems of stimulated growth (Allfrey, 1970). Quantitative analyses of nuclear proteins during liver regeneration after partial hepatectomy have been mainly made on rats (Evans et al., 1962; Thaler and Villee, 1967; Bannai and Terayama, 1969; Orlova and Rodionov, 1970). Therefore, in this preliminary study on mouse liver regeneration, the nuclear content of neutral, basic, acidic and residual proteins has been analysed. The results have been compared to those found after a sham operation. A fraction of the chromatin associated nuclear proteins in normal liver has been isolated and compared to the mouse serum proteins using electrophoresis.

#### Experimental procedure

Animals. Young male and female C3H-mice from an inbred strain kept at the institute were used for the experiments. The weights of the mice and their housing and feeding have been described earlier (Lewan, 1971).

Partial hepatectomy. Partial hepatectomy was performed essentially according to Higgins and Anderson (1931) at a constant time in the morning as described previously (Heby and Lewan, 1971).

Isolation of nuclei. The nuclei were isolated according to the method of Blobel and Potter (1966). 1 gram of liver was minced with scissors and homogenized in 3 ml of 0.25M sucrose in a Tris-KCl-MgCl<sub>2</sub> buffer, pH 7.0, using a motor-driven glass-teflon homogenizer. The homogenate was mixed with 9 ml of 2.3M sucrose in the TKM-buffer and filtered through nylon net into centrifuge tubes. It was underlayered with 2.3M sucrose and centrifuged at 80 000  $g_{av}$  for 1 hour in a MSE superspeed 50 centrifuge. The crude nuclear pellet was used for extraction of proteins. All preparations were performed at +4°C.



Protein extractions for quantitative estimations. The nuclear pellet was resuspended by homogenization in TKM-buffer. The homogenate was centrifuged at 25 000 g for 30 minutes. The supernatant was used for the estimation of the amount of neutral soluble nuclear protein. The pellet was extracted once with 0.25M HCl and once with 0.05M HCl. The pooled supernatants were used for the estimation of the amount of acid soluble nuclear protein.

The pellet resulting from centrifugation was extracted with 0.05M KOH-5M Urea-0.05M mercaptoethanol by repeated stirring for 30 min. After recentrifugation the pellet was resuspended in TKM-buffer and the suspension was used for determination of the amount of residual nuclear protein.

The amount of protein in each fraction per mg of DNA in the nuclear pellet after ultracentrifugation was calculated.

Quantitative protein analyses. The amount of protein in the suspension of the crude nuclear pellet, in the TKM-extract, in the pooled HCl extracts and in the residual suspension was determined according to Lowry (1951). The difference between the amount of protein in the crude nuclear pellet and the sum of the protein found in the TKM-extract, the HCl-extract and the residual suspension gave the amount of protein in the KOH-extract. Albumin (Sigma) fraction V dissolved in water was used as a standard. In the acid soluble fraction the values measured were corrected for the effect of HCl.

Nucleic acid analysis. The amounts of DNA and RNA present in the suspension of the crude nuclear pellet after ultracentrifugation were determined according to Burton (1956) as described earlier (Heby and Lewan, 1971). Calf thymus DNA (Sigma) was used as a standard.

Protein extraction for electrophoresis. The crude nuclear pellets obtained from 8 normal livers were pooled and extracted for proteins by a modification of the method used by Benjamin and Gelhorn (1968).



The nuclei were extracted first with 0.1M tris-0.14M NaCl-0.01M EDTA, pH 6.5 and then with 0.15M HCl and 0.05M HCl. The remaining pellet was suspended in 2 ml 4M CsCl in 0.02M lysine buffer-0.005M EDTA-0.01M mercaptoethanol, pH 11.6. The suspension was centrifuged at 180 000 g in a MSE superspeed 50 centrifuge for 48 hours. The upper half of the supernatant was concentrated by overnight dialysis with negative pressure against 0.05M tris-buffer, pH 7.5, and then used for electrophoresis.

Electrophoretic methods. Disc electrophoresis was performed on polyacrylamide gels according to a modification of the system of Davis (1964). Thus by using diethanolamine as a buffer in stock solution A the pH was increased to 9.5. The pH of stock solution B was increased to 7.6 with tris buffer. 0.2M  $\beta$ -alanin-diethanolamine, pH 9.0, was used as the electrode buffer. Electrophoresis was carried out at 100 V and 6mA per gel for 1 hour. The gels were stained using Amidoblack and destained electrophoretically in 7% HAc.

### Results

Nuclear RNA. A tendency was found towards increased  $\frac{\text{RNA}}{\text{DNA}}$  ratios in the nuclei after partial hepatectomy. After a shamoperation the nuclear  $\frac{\text{RNA}}{\text{DNA}}$  ratios showed a decrease, Fig. 1A.

Nuclear protein. After hepatectomy the total amount of nuclear protein measured per DNA, tended to increase. A sham operation possibly lowered the amount of nuclear protein, Fig. 2B.

Fractionation of the nuclear protein revealed that the increased  $\frac{\text{protein}}{\text{DNA}}$  ratio depended upon increased amounts of protein in the neutral and alkali soluble fractions (Figs, C, D, E, F).

Changes in nuclear RNA and protein caused by hepatectomy. The extent to which the changes in the RNA/DNA and protein/DNA ratios may be referred to hepatectomy alone is shown in Fig. 2. These curves were calculated as the differences between the ratios for shamoperated and hepatectomized animals. A hepatectomy seemingly caused an accumulation of RNA and also of





total protein relative to DNA. The accumulated protein should mainly be constituted by neutral soluble and alkali soluble protein.

Electrophoresis. When chromatin extracted of neutral and acid soluble protein was dissociated into DNA and protein by CsCl gradient centrifugation, some protein fractions were obtained having electrophoretic properties similar to those of serum proteins, Fig. 3.

### Discussion

During the premitotic period of liver regeneration analysed the nuclear volume increased (Grundmann and Bach, 1960) and the RNA synthetic activity was elevated (Bucher and Swaffield, 1969). The nuclei started to enter DNA synthesis between 30 and 40 hours (Jardetzky et al., 1956; Bade et al., 1966) and entered mitosis between 36 and 48 hours after hepatectomy (Wilson et al., 1953; Russo and Echave Llanos, 1964; Heby and Lewan, 1971).

The accumulation of RNA in the nuclei, indicated to be caused by hepatectomy in this study, occurred when the nucleolar concentration of RNA is known to be elevated (Stowell et al., 1948) and when the total liver RNA/DNA ratio is also increased (Novikoff and Potter, 1948; Lewan, 1972).

The rate of synthesis of ribosomal units relative to their transport to the cytoplasm may bring about an accumulation of RNA in the nuclei (Chadhuri and Lieberman, 1968; Church and McCarthy, 1967; Rizzo and Webb, 1971). In chromatin isolated from regenerating rat liver, increased or decreased RNA/DNA ratios were indicated (Thaler and Villee, 1967; Tidwell, 1968).

The accumulation of protein within the nuclei, indicated after hepatectomy (Fig. 1B), occurred when the liver protein concentration was below the normal level (Lewan, 1972). During this period the liver cells secrete proteins at an elevated rate (Schreiber et al., 1971). The concentration of protein within the nuclei also decreases due to influx of water (Hofmeier and Grundmann, 1962) and fat (Brock et al., 1952). Relative to DNA, however, there may be an accumulation in the nuclei of mainly neutral and





alkali soluble protein (Fig. 2). In other studies increased (Evans et al., 1962; Orlova, 1970) or decreased (Thaler and Vिलlee, 1967) amounts of acid soluble protein have been found in nuclei after hepatectomy, and rather constant amounts of non-histone protein (Evans et al., 1962; Thaler and Vилlee, 1967; Bannai and Terayama, 1968). However, increased amounts of acidic proteins have been found in active chromatin as compared to repressed (Frenster et al., 1963), and the amount of active chromatin actually increases during liver regeneration (Dolbeare and Koenig, 1970). Acidic protein in the neutral soluble and in the alkali soluble nuclear protein fractions is supposed to be active at gene regulation (Teng et al., 1971; Stein and Baserga, 1970; Kostraba and Wang, 1970).

The presence in the liver chromatin of proteins with electrophoretic properties similar to those of serum proteins does not seem to be brought about by cytoplasmic contamination. The isolated nuclei appeared uncontaminated at microscopic examination and cytoplasmic proteins should have been extracted during the fractionation procedure. Similar results are also indicated by the electrophoretic results in other studies (Benjamin and Gelhorn, 1968; Wang, 1969; Kostraba and Wang, 1970; Gronow and Griffiths, 1971; Teng et al., 1971). Still, the chromatin acidic proteins have been considered to represent a separate class of proteins from those remaining in the cytoplasm (Wang, 1969; Kostraba and Wang, 1970; Baserga and Stein, 1971).

In general the results presented here should be interpreted with caution. It is known that the proportion between parenchymal and non-parenchymal nuclei changes during regeneration (Yokoyama et al., 1953) and a changing selection of nuclei may occur during the preparation with the method used (Lewan, unpublished). However, the non-parenchymal nuclear chromatin constitutes a small proportion of the total liver chromatin. During the period of liver regeneration analysed the cytoplasm is rich in lipids, which may increase the adherence of cytoplasmic RNA and protein to the nuclear membrane (Barton et al., 1971).



Thus the increase in nuclear RNA and protein observed in this study may reflect a structural change in the liver tissue during regeneration rather than a real accumulation within the nuclei. No adhering extranucleolar layer was however found when isolated nuclei were observed under the microscope.



Fig. 1. RNA and protein per DNA measured as weight per weight in liver nuclei isolated from normal liver and livers after sham operation (○-○-○-○-○) and after hepatectomy (○-○-○-○-○). Each point represents nuclei from the liver in one normal or sham operated mouse or nuclei from pooled livers from 3 hepatectomized mice.

Fig. 2. Changes in RNA/DNA and protein/DNA ratios caused by hepatectomy alone. The difference between the curves for respective ratio in hepatectomized and sham operated mice in Fig. 1 has been added to the ratio in normal mice. Semilogarithmic scale.

Fig. 3. Schematic drawings of polyacryl-amide gels containing A) serum proteins and B) a fraction of chromatin acidic proteins.



Fig. 1

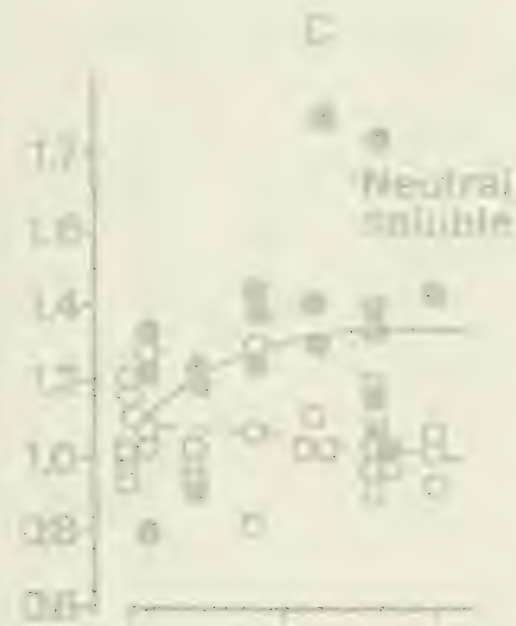
RNA per DNA w/w



Protein per DNA w/w



Protein per DNA w/w



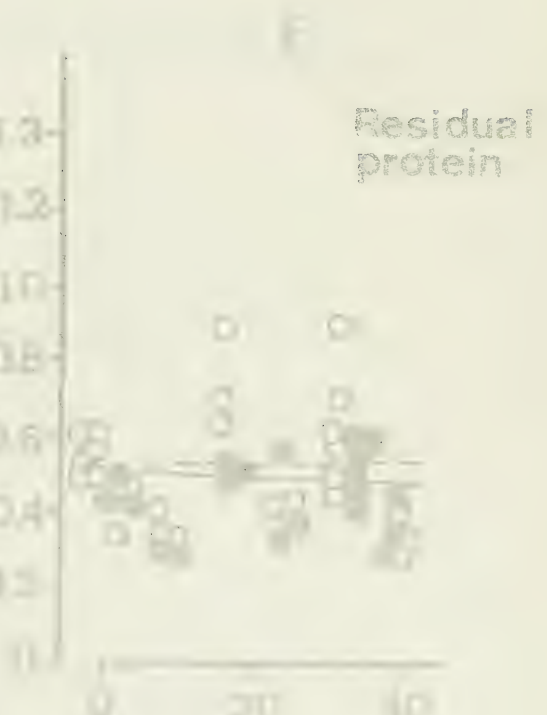
Protein per DNA w/w



Protein per DNA w/w



Protein per DNA w/w



Hours after operation





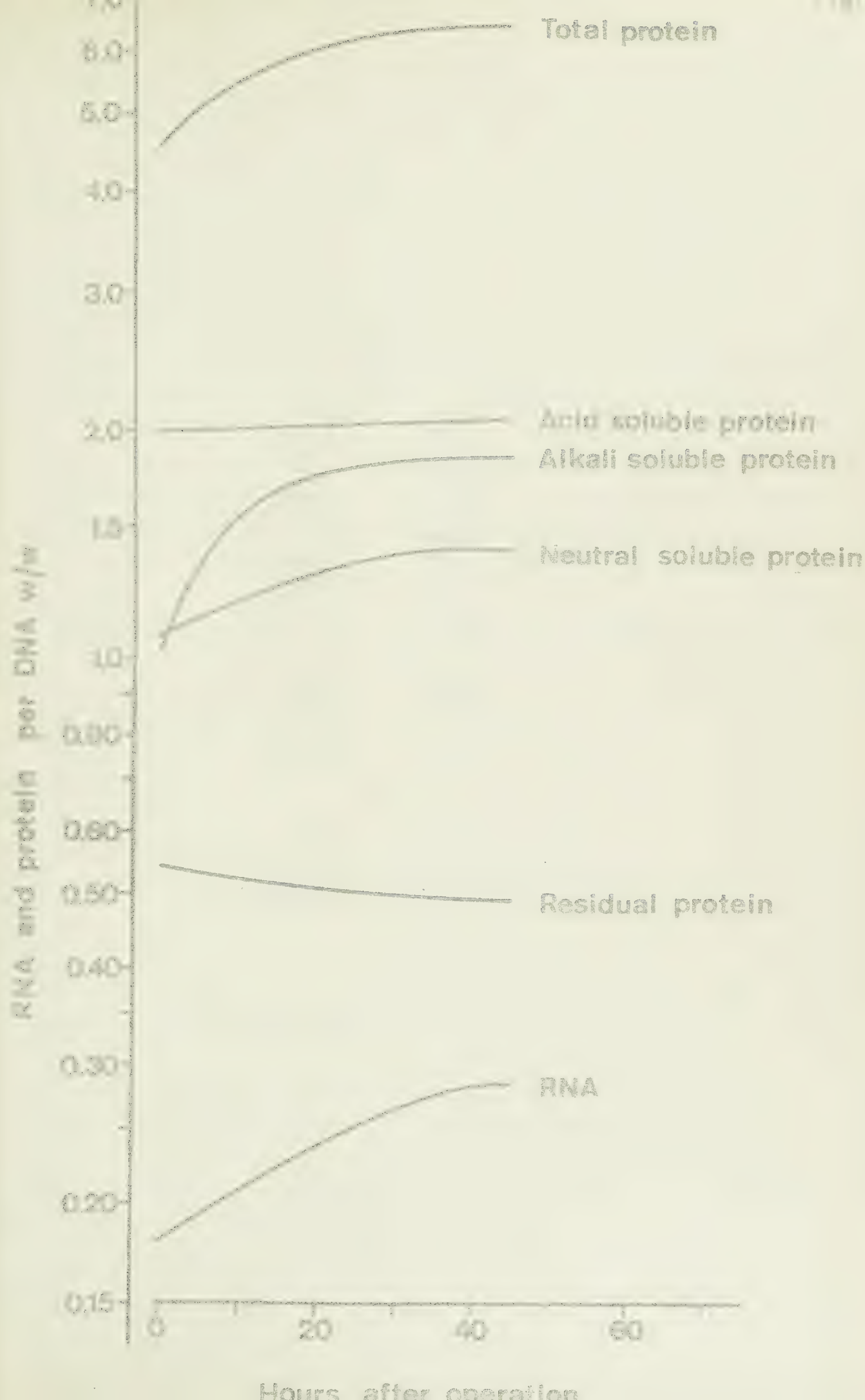


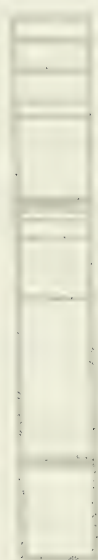


Fig. 3



A

Serum  
protein



B

Chromatin  
protein



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